Vesicles in Nature and the Laboratory

Fernandez-Trillo, Francisco; Grover, Liam M.; Stephenson-Brown, Alex; Harrison, Paul; Mendes, Paula M.

DOI:
10.1002/anie.201607825

Document Version
Peer reviewed version

Citation for published version (Harvard):
Fernandez-Trillo, F, Grover, LM, Stephenson-Brown, A, Harrison, P & Mendes, PM 2017, 'Vesicles in Nature and the Laboratory: Elucidation of Their Biological Properties and Synthesis of Increasingly Complex Synthetic Vesicles', *Angewandte Chemie (International Edition)* , vol. 56, no. 12, pp. 203–229.
https://doi.org/10.1002/anie.201607825

A Journal of the Gesellschaft Deutscher Chemiker

Angewandte International Edition Chemie

GDCh

www.angewandte.org

Accepted Article

Title: Vesicles and their multiple facets: underpinning biological and synthetic progress

Authors: Paula M. Mendes, Francisco Fernandez-Trillo, Liam Grover, Alex Stephenson-Brown, and Paul Harrison

To be cited as: *Angew. Chem. Int. Ed.* 10.1002/anie.201607825
Angew. Chem. 10.1002/ange.201607825

Link to VoR: http://dx.doi.org/10.1002/anie.201607825
http://dx.doi.org/10.1002/ange.201607825

WILEY-VCH

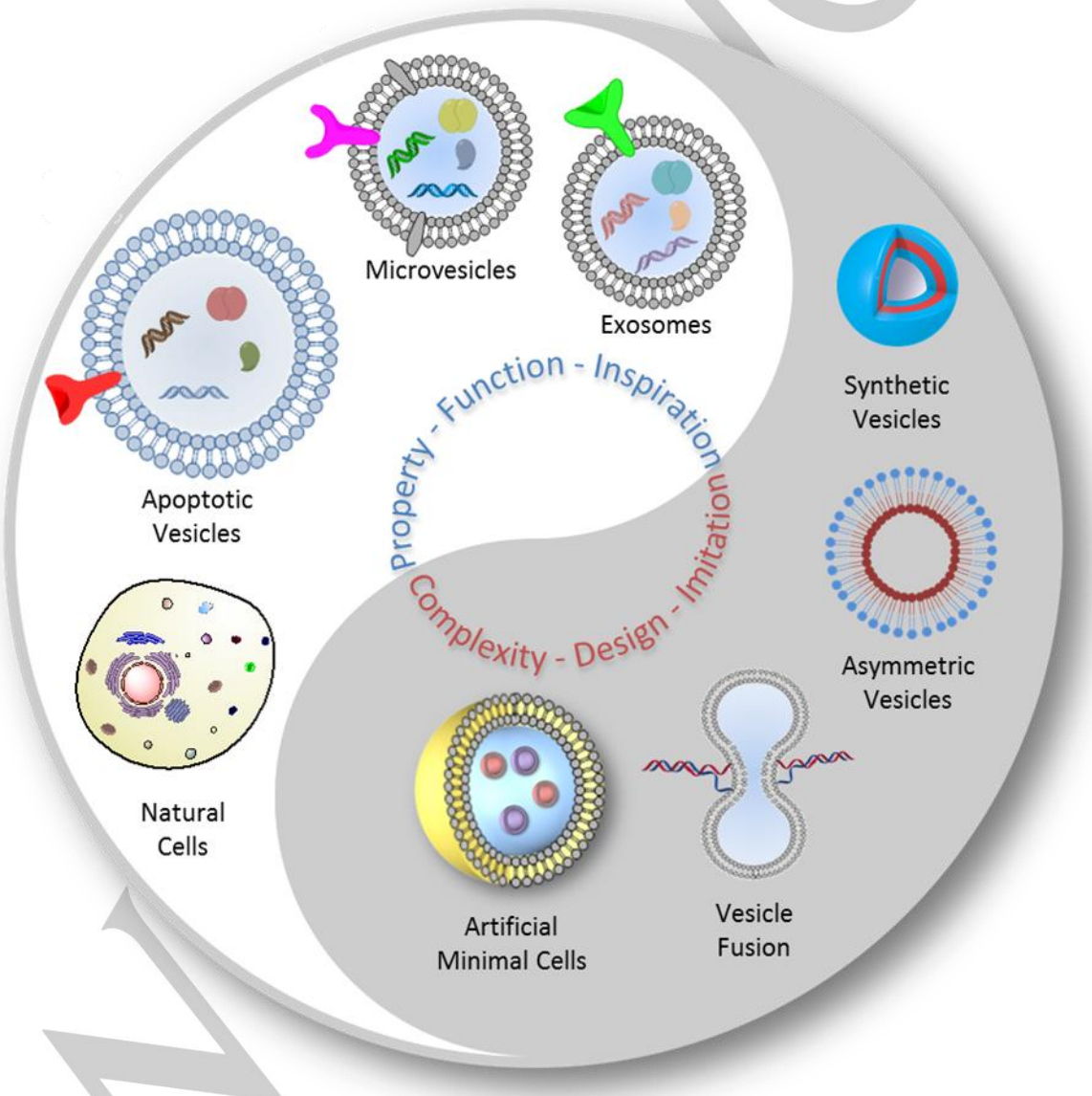
10.1002/anie.201607825

WILEY-VCH

Vesicles and their multiple facets: underpinning biological and synthetic progress

Francisco Fernandez-Trillo,*[a] Liam M Grover,*[b] Alex Stephenson-Brown,[b] Paul Harrison,[c] Paula M Mendes,*[b]

[a]School of Chemistry, [b]School of Chemical Engineering and [c]Institute of Inflammation and Ageing (IIA), University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK



The important role of vesicles in many aspects of cell function is well recognized but only recently have sophisticated imaging techniques begun to reveal their ubiquity in nature. While we further our understanding of the biological properties of vesicles and their physiological functions, increasingly elegantly designed artificial vesicles are being reported for a wide range of technological applications and basic studies. Herein, we bring together both the biological and synthetic state-of-the-art on vesicles and place their biological features in the context of recent synthetic developments, providing a unique view of these complex and rapidly developing fields. The perspectives on the challenges and opportunities for future biological and synthetic progress on vesicles are also presented.

1. Introduction

While vesicles have been known to exist for more than 50 years,[2, 3] it was in the early 1990s when we witnessed the breakneck pace of scientific advancements in vesicle biology. Recent innovation[9] in analytical techniques for imaging and detection of hydrated vesicular structures have tremendously expanded our understanding of how the chemical structure and function of vesicles contribute to biological roles as diverse as compartmentalization, storage and molecular trafficking. Vesicle trafficking is inextricably linked with processes of secretion (exocytosis) and uptake (phagocytosis and endocytosis) and, among other functions, allows for transit of signaling molecules that mediate cellular communication.[12] In addition, vital roles in a wide variety of physiological and pathological processes are beginning to be recognized, ranging from signaling in the brain, regulation of immunity, coagulation, angiogenesis and cancer progression.[12, 13] Recent findings also indicate that signals shuttled by stem cell-derived extracellular vesicles (EV) may be critical in the maintenance of stemness or differentiation, as well as in stem cell-mediated tissue repair after injury.[17]

The distinct and diverse range of vesicle functions is enabled by exquisite chemical and structural variation that ultimately allows for the guidance, release or deposition of their contents in a specific location intra- or extra-cellularly. It is this deeper understanding of their morphological, structural and biological characteristics that is providing us with the tantalizing possibility of tailoring structure/chemistry such that they may be used in a multitude of biological and non-biological applications. In parallel, our knowledge and expertise in synthetic, supramolecular, colloidal and biological chemistry has progressed significantly in recent years and as such our capacity to mimic nature's ability to build sophisticated vesicular systems has improved. We can now build artificial vesicles that show some or many of the traits found in natural vesicles and cells, including membrane compositional heterogeneity, membrane recognition, and/or compartmentalization.

Owing to the high complexity of biological systems, synthetic vesicles have served as highly valuable model systems for the analysis of diverse biophysical processes occurring at cell membranes, including membrane permeability, cellular transport, endo- or exocytosis and fusion of membranes.[18, 19, 20] Additionally, vesicles are being tailored to allow both targeted delivery and sustained release, and have been applied as vehicles for delivery of both hydrophobic drugs, which are incorporated into the membrane, and hydrophilic drugs, which are encapsulated in the interior aqueous compartment.[21, 22, 23] Furthermore, bottom-up approaches are being actively pursued to engineering more complex vesicle-based systems and construct artificial minimal cells[24] that mimic functions of natural cells, including gene expression, membrane transport, subcellular localization and biochemical reactions. The minimal-cell model system is meant to represent the hypothetical precursor structures of early living cells in early evolution, creating new opportunities for insights into the origin of life.[25, 26] Concurrently, with recent advances in the construction of vesicle-based minimal cell analogues, innovative platforms for biotechnology and biomedical applications are being introduced.

While these two fields of research – biological vesicles and artificial vesicles – have much to contribute to each other, and despite the vast amount of progress in these areas, they are yet to be discussed together critically in a single review. It is therefore our aim in this review to bring together our knowledge of the specialized structure and function of diverse biological vesicles and the recent advances in the synthesis of vesicle systems from synthetic and biological building blocks. We will first describe our current understanding of biological vesicles, focussing on extracellular vesicles and their classification. Extracellular vesicles, similarly to most synthetic vesicles, are generated to function outside of the cell, laying out a common ground for joint discussion of their properties and functions. It is beyond the scope of this review to delve into the different aspects of intracellular vesicles (i.e. peroxisomes and lysosomes) and the reader is directed to recent excellent reviews on the subject.[27, 28, 29]

In the following sections, we discuss different properties of biological and synthetic vesicles, namely membrane compositional heterogeneity, membrane binding and downstream events compartmentalization, internalized chemical transformations and growth and self-replication. Besides providing insights into their functional importance in a biological setting, the discussion is also focussed on how such properties are achieved biologically and synthetically. Where applicable, we highlight how biological systems have inspired synthetic systems and how synthetic systems have been providing insights into structure and function of biological vesicles. A brief look at the current status and the future outlook of the biological and artificial vesicle field concludes this review.

Francisco Fernandez Trillo (Paco) is the John Evans Fellow of Nanotechnology at the University of Birmingham (UK) developing Biomedical Applications of Nanomaterials. Prior to this he received his PhD in Organic Chemistry in 2004 from the University of Santiago de Compostela (Spain). This was followed by post-doctoral positions, both in the UK (Durham and Nottingham) and Spain (Santiago de Compostela) working on polymeric nanomaterials for biocatalysis, cell recognition and synthetic biology. Paco now leads a diverse research group working at the interface between organic chemistry, polymer science and nanotechnology.

Liam M Grover is a Professor of Biomaterials Science at the University of Birmingham. He received his PhD at the University of Birmingham, prior to undertaking postdoctoral training as a CIHR Skeletal Health Scholar at McGill University (Canada). Professor Grover has a strong interest in ossification and has worked for the past ten years to develop materials and therapies that interact with the bone formation process, with the aim of either stimulating it or preventing pathological bone formation. He has a major interest in how extracellular vesicles mediate the process of ossification and calcification and is working on ways to harness these to prevent non-union and pathological ossification.

Paula Mendes received her MSc (1997) and PhD (2002) degrees in Chemical Engineering from the University of Porto, Portugal. She undertook post-doctoral research at the University of Birmingham, UK (2002–04) and at the University of California, Los Angeles, USA (2004–06). In 2013, she became a Professor of Advanced Materials and Nanotechnology and an EPSRC Leadership Fellow in the School of Chemical Engineering, University of Birmingham, UK. Her research interests lies in the development of novel methods for controlling the structure and functionality of materials at the molecular and nanometer scales and their application in biology and medicine.

2. Extracellular vesicles

Although it has been known that extracellular vesicles play an important role in the clotting of blood since the 1940's[30] and in the controlled mineralization of bone since 1969,[31] until recently the scientific community had not appreciated the full breadth of processes in which extracellular vesicles were central. Vesicles have now been shown to mediate DNA, mRNA and microRNA transfer,[32, 33, 34] promote[35] and inhibit inflammatory processes,[36] mediate mineralization and facilitate para- and juxtacrine communication.[37, 38] Recent work has strongly implied that certain beneficial effects following stem cell treatment may occur as a consequence of the secretion of exosomes by mesenchymal stem cells.[39, 40] The prospect of harnessing the therapeutic benefits of mesenchymal stem cells without the risks of carcinogenesis and immunological response has generated a considerable amount of academic and commercial interest in these exosomes and other cell derived vesicles.[41] Given that such vesicles may be classified as medicines, there is now an important concerted effort between academics and industry alike, to characterize vesicular markers, properties and potency so that they may be translated into the clinic.

Extracellular vesicles (EVs) also have enormous potential as biomarkers.[42, 43] Measurement of EVs within biological fluids provides a non-invasive "liquid biopsy" of health and disease. Changes in EV size, concentration, protein and RNA content have essentially been described in all major diseases (e.g. cancer, cardiovascular disease, autoimmunity and disorders of pregnancy).[42, 43] Biophysical approaches are now available for measuring EV, including the application of nanoparticle tracking analysis, dynamic light scattering and tunable resistive pulse sensing amongst others.[44] These allow accurate measurement of EV number and size distributions but are still limited in measuring of phenotypes.

Major challenges that remain in this area are their characterization and categorization of vesicles of biological origin. At present, a surfeit of terms has been used to describe what are very similar structures with overlapping function, size and origins (endosome, exosome, microvesicle, ectosome, matrix vesicle, microparticle, shedding vesicles).[42, 45, 46]

2.1. Vesicle classification

With the growth of the research focused on EVs and a recent explosion in the literature, there has been a significant effort to harmonize the definition of the different forms of vesicles.[47] The current terminology and definitions associated with extracellular vesicles are given in **Table 1**. An important observation is that the size ranges for biogenic vesicles lies on a continuum and as such it is frequently extremely challenging to classify vesicle origin and function.

Table 1. A summary of the properties of the three types of cell-derived vesicle with distinct biogenic origins.

Vesicle type	Size range (nm)	Distinctive Features	Origin
Exosome	30-100	Presence of specific membrane receptors: tetraspanins (CD9, CD63), Alix, flotillin-1 and Tsg101	Derived from the ESCRT pathway and fusion of the MVB with the outer membrane
Microvesicles	20-800	Comparatively high surface phosphatidyl-serine content	Budded directly off the surface of the cell membrane
Apoptotic vesicles/bodies	500-2000	Can contain densely packed organelles and may contain DNA	Blebbed from the surface of the cell membrane, often during apoptosis

Efforts are currently being made to identify markers that can facilitate the identification of EVs from different sources.[48] There is now a consensus emerging that there are three main classes of vesicle with differing membrane structure: Exosomes, micro-vesicles and apoptotic vesicles. Each of these classes is distinct in the manner that they are formed, which leads to them having very distinct membrane properties and functions. Importantly, the identification of surface markers for these vesicles has allowed for researchers to begin more systematically classifying these structures, which are found in a multitude of biological fluids.

2.2. Exosomes

Despite an overlap in diameter, microvesicles and exosomes have distinct markers and a clearly contrasting mechanism of biogenesis. The formation of exosomes occurs through the ESCRT (Endothelial Sorting Complexes Required for Transport) pathway, whereby particles or macro-molecules pass through the cell membrane and are encapsulated within an early endosome.[49] The early endosome is marked by the ESCRT complex and migrates to an aggregation of vesicles found within the cell known as the Multi-Vesicular Body (MVB) (**Figure 1**).[50] The development of local heterogeneities within the membrane structure of the vesicle provokes deformation and invagination of the membrane and ultimately the formation of an interluminal vesicle (ILV).[51] The MVB is anchored to a network of micro-tubules within the cell on which the MVBs may be transported directly to a specific location. The movement of these vesicles through the cell occurs via the molecular motors dynein and kinesin,[52] which are thought to facilitate the movement of the vesicles to the periphery of the nucleus or the cell membrane, respectively. The mechanism of movement and secretion, however, is significantly more complex and many of its intricacies remain unknown. Researchers have, however, identified that levels of cholesterol within the vesicle correspond strongly to vesicular movement, with unconstrained concentration of cholesterol within the MVB preventing migration to the cell membrane.[53] The movement of the MVBs to the cell periphery are strongly associated with the Rab proteins and Rab27 has been shown to be critical to this process, but is not responsible for the fusion of the MVBs with the cell membrane.[54] Other Rab proteins have been found on the surface of recovered exosomes and as such are implicated with the membrane fusion process (Rab35 and Rab11).[55] Another family of proteins known as SNAREs enhance the association of the MVBs with the internal leaflet of the cell membrane (**Figure 1**).[56] A subsequent intracellular influx of calcium ions initiates fusion and the ILVs within the cell may be released into the extracellular fluid.

Exosomes contain a population of membrane and cytosolic proteins, lipids and RNA that vary in part according to the cells from which they originate.[57] They are characterized by the presence of lipid rafts, which allow for sorting of raft-associated proteins, such as follitin and tetraspanins, and regulation of signaling processes. Exosome composition and organization enable them to play a pivotal role in cell to cell communication, particularly between the far distance cells in the body. Exosomes are not only responsible for triggering downstream signaling but they also specifically target the recipient cells and deliver proteins and RNA to them.[57]

2.3. Microvesicles

Rather than being derived from the ECSRT, microvesicles bud directly from the cell membrane. They are also known as micro-particles, shedding vesicles, ectosomes and exovesicles.[42] They form following the influx of calcium ions into the cell, which causes the reorganization of the cell cytoskeleton and results in the formation of nanodomains within the cell membrane. As they are derived directly from the cell membrane, these vesicles exhibit high concentrations of phosphatidyl-serine (PS) and cholesterol. During the formation of the microvesicles,

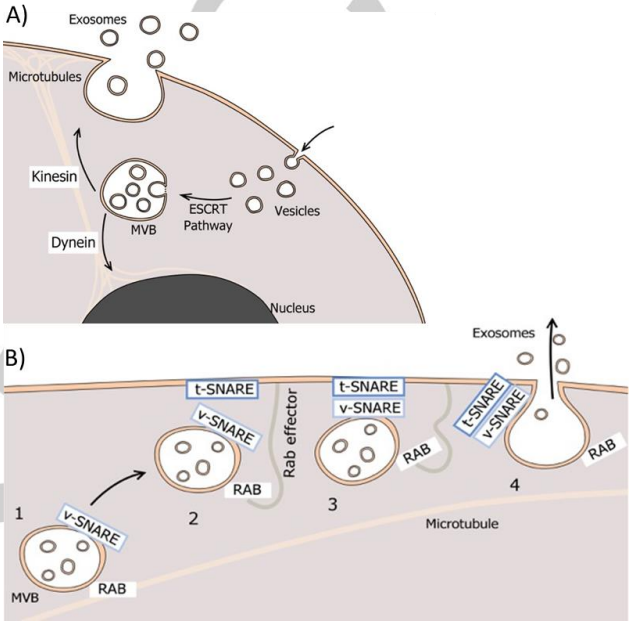


Figure 1. Exosomes are generated through the ESCRT (Endothelial Sorting Complexes Required for Transport) pathway. Briefly, vesicles are formed after the absorption of extracellular objects through the cell membrane. A) These vesicles are transported to a MVB (multivesicular body) and may then be transported to the nucleus or to the cell membrane, this movement is mediated by the molecular motors dynein and kinesin. B) At the membrane, Rab and SNARE proteins mediate attachment to the internal leaflet of the membrane, after which they merge with the membrane and release the exosomes into the extracellular space.

flippase and scramblase activity is modified, which inverts the inner leaflet of the membrane and exposes PS. The enzymes flippase, floppase and scramblase are actively involved in creating and maintaining asymmetry in the cell membrane by enabling the movement of lipid molecules between the internal and external leaflets of the membranes. Modifying the balance in molecular transport through the membrane reduces the stability of the cell membrane. This, in addition to the action of calpain, which cleaves the attachment of the cytoskeleton to the membrane cause the budding of vesicles from the cell membrane. During the formation of microvesicles, proteins from the cell membrane and within the cell are collected and released associated with the microvesicles.[58, 59] Unlike exosomes, the composition of microvesicles is not so well characterized but their protein enrichment on cytokines, chemokines, matrix metalloproteinases and integrins have been reported.[61] Nevertheless, protein composition depends on cell type of origin. Microvesicles, in common with exosomes, are intracellular protein and RNA

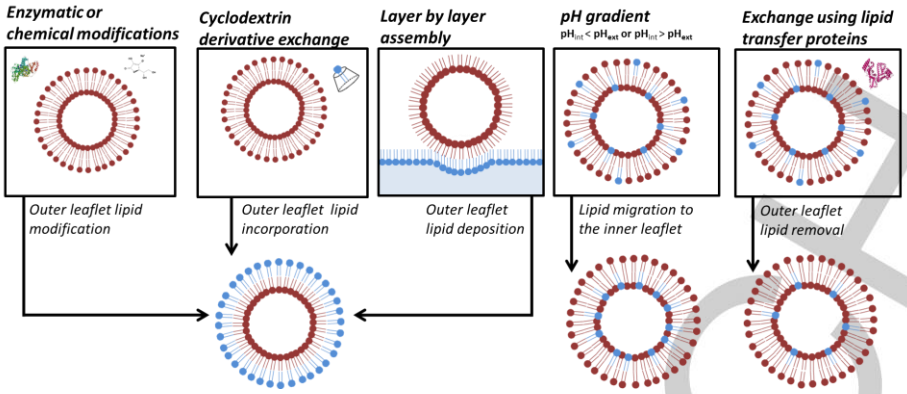


Figure 2. Schematic diagram of some of the reported strategies to generate asymmetric vesicles, namely enzymatic[1] and chemical[4] modifications on the outer leaflet, outer leaflet lipid exchange using cyclodextrin derivatives,[5, 6] layer by layer assembly on emulsified water droplets[8] and microfluidic droplets,[11] pH gradient imposed across the membrane[14] and exchange using lipid transfer proteins, including removal of outer leaflet lipids.[15, 16]

transporters and share the capability to reprogram the recipient cell.[61] For instance, microvesicles are important carriers of cytokines and chemokines that can induce subsequent cell and tissue response, which can have a multitude of therapeutic or pathological effects.[46, 60]

2.4. Apoptotic vesicles

As shown in **Table 1**, apoptotic vesicles or apoptotic bodies may be considerably larger than exosomes or microvesicles. Similarly to microvesicles they form directly from the cell membrane during the process of apoptosis. In addition to cell membrane and cytosolic proteins, apoptotic vesicles contain DNA. They also exhibit exposed PS on the surface of the membrane and, in contrast to both microvesicles and exosomes, have a membrane of sufficient permeability to allow for staining of the intervesicular DNA using propidium iodide. The predominant role of these vesicles is as yet unknown, but it has been hypothesized that they may be responsible for horizontal gene transfer between cells.[62]

3. Membrane compositional heterogeneity

Lateral (heterogeneities at one leaflet of the membrane, termed lipid rafts) and transverse (different constituents on the inner and outer leaflets of the bilayer) asymmetry in cell membranes play key roles in many cellular events. Lipid rafts are dynamic submicroscopic assemblies, more ordered and tightly packed than the surrounding bilayer and involved in cellular processes such as adhesion, budding and signaling.[63, 64] Cell membranes are also characterized by transversal asymmetry with specific lipids enriched in the extracellular leaflet, whereas others, namely PS and phosphatidylethanolamine (PE) are primarily localized in the cytoplasmic leaflet. Transverse asymmetry, which is maintained by a family of membrane-bound transport proteins called phospholipid translocases, is required for normal membrane function and disruption of this asymmetry is a biochemical feature in apoptosis, platelet activation and cell fusion.[65, 66]

Membrane compositional heterogeneity have been also reported in EVs, recognized to be related with the process of vesicle formation and expected to play a key role in trafficking and signaling, and thus in their regulatory mechanisms. The composition of EV membranes does not reflect that of the parent cell membrane but are enriched or depleted in particular lipids and proteins of their parent cell**s**.[67] Exosomes, which have been the most studied by lipidomics, exhibit lipid raft-like domains. Exosome composition is enriched with lipids that are critical for the maintenance of rafts, such as cholesterol, sphingolipids, ceramide, and glycerophospholipids with long and saturated fatty-acyl chains.[68] Many raft-associated proteins are also present in exosomes, including tetraspanins, GPI-anchored proteins Src tyrosine kinases and proteins containing prohibitin domains.[67, 69] Owing to the high content of raft-associated lipids and proteins and their organization, exosomal membranes exhibit greater rigidity than cell plasma membranes. The rigidity is pH-dependent and increases from pH 5 to pH 7.[70] The elevated structural rigidity is anticipated to enhance the membrane fusion with the plasma membrane of recipient cells at physiological pH and prevent lipolytic or proteolytic degradation of exosomes while in circulation. It also accounts for their stability under various storage conditions for up to 90 days.[71]

EVs are also often typified by an increased rate of transbilayer movements of phospholipids (i.e. flip-flop) as compared to the plasma membranes of parent cells.[70, 72] It can result in symmetry in lipid distribution between the two membrane leaflets and PS externalization, whereas its asymmetric distribution in the inner leaflet of plasma membrane is well established. The loss of asymmetry is likely due to the lack of translocase activity. While absence of lipid translocases has been reported,[73, 74] phospholipid asymmetry in some EVs is still achieved and might be explained by their interactions with cytosolic protein domains.[74] EVs are diverse in composition and organization, and currently more studies are necessary to understand their structure and, indeed, its correlation with properties and function. It will ultimately, for instance, inform the formulation of specific and effective vesicle-based therapeutics, i.e. vesicles with specific target recognition, no cargo leakage, better stability and longer circulation times.

While we further our understanding of how EVs display a large repertoire of biomolecules that affect their overall properties and functions, important progress has also been made in creating lateral and transversal heterogeneity in synthetic vesicles. In artificial systems, lateral asymmetry has been achieved using mixtures of lipids with different phase transition temperatures. This asymmetry results in phase separation and genera-

tion of different lateral phases, including liquid disordered, liquid ordered and solid-like gel phases.[75] Lipid mixtures can therefore be developed that phase separate into two or more of these coexisting phase domains, creating lipid rafts.[76] In a study conducted by Yanagisawa and co-workers,[77] lipid phase separation was explored in conjunction with differences in osmotic pressure to induce complex vesicle shape transformations that were followed by domain budding.

Vesicles exhibiting Janus-like morphology have also been constructed in a way that allowed the local confinement of DNA.[78, 79] Inspired by how membrane proteins are targeted to raft domains through palmitoylation (i.e. the post-translational addition of palmitic acid), Arbuzova and co-workers[79] were able to palmitoylate a DNA-recognizing peptide nucleic acid (PNA) with the aim of exclusively partitioning the PNA into the liquid-ordered domains of liquid-liquid phase separated giant unilamellar vesicles (GUVs). This strategy was highly effective, allowing the combination of specific partitioning of lipophilic DNA and PNA molecules into defined membrane environments with the reversible temperature-dependent intermixing of laterally separated membrane domains.

In contrast to the limited number of methods on how to achieve lateral asymmetry, a variety of strategies have been reported over the years to construct vesicles with transverse lipid asymmetry (**Figure 2**). It is noteworthy that these strategies are associated with various degrees of control of asymmetry and scope of components that can be used to achieve an asymmetric bilayer. Early examples of synthetic asymmetric vesicles relied on enzymatic[1] and chemical[4] modifications of external lipids and the capability of lipid transfer proteins to promote lipid redistribution.[15, 16] Transmembrane pH gradients have also been exploited successfully to induce transverse asymmetry in vesicles partly composed of lipids bearing weak acid or weak base headgroups.[14] The properties of this amphiphilic system have been also fine-tuned to create compositional asymmetry in the two leaflets. Membrane-spanning bola-amphiphiles with two headgroups of different sizes have been reported to self-assemble into asymmetric vesicles via steric effects.[80] In a conceptually similar fashion, amphiphilic asymmetric block copolymers can also be used to create vesicles with asymmetric membranes.[81, 82, 83] Taking ABC copolymers, where both outer blocks (A and C) are hydrophilic and the central (B) block hydrophobic, as an example, the chemical nature of the polymer chains expressed at the interior or exterior of the vesicle can be controlled by the relative size of the hydrophilic blocks. In general, the longer amphiphilic block (A or C) is segregated to the exterior of the vesicle, while the shorter one is directed to the vesicle's interior to minimize the interfacial tension and enhance the vesicle curvature.[82, 84]

Recently, Kimura and co-workers have harnessed the properties of amphiphilic block polypeptides to construct peptide-based asymmetric vesicles.[85] Key for achieving asymmetry was the use of right- and left-handed helical polypeptides, which were capable of forming a stereocomplex and could be arranged alternately in the vesicular membrane (**Figure 3**). Furthermore, dipole-dipole interactions between the hydrophobic helices allowed an interdigitated helix packing of the stereocomplex. Using this antiparallel arrangement and introducing steric effects, control over the molecular orientation of two host right- and left-handed helical polypeptides was achieved. Further selective modification of either the outer or inner surface of the binary vesicle was possible by inclusion of a third guest helical polypeptide in the bilayer, in which the helix sense (right- or left-handed) defined the orientation of the polypeptide in the membrane.

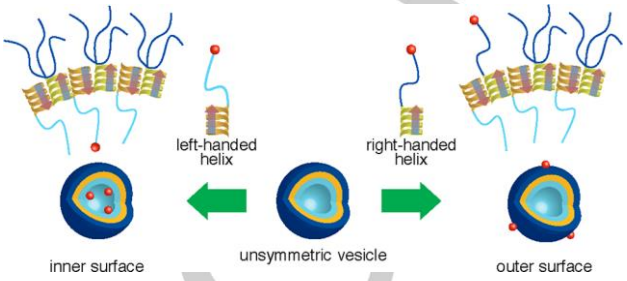


Figure 3. Schematic representation of a peptide-based asymmetric vesicle formed as a result of helix dipole, stereocomplex and steric effects. Modification at the outer or inner surface can be performed by selecting either a right-handed or left-handed helical peptide.

Strategies have also been devised based on the ability of cyclodextrin (CD) derivatives to bind phospholipids and to exchange them with a pre-formed lipid bilayer.[5, 6] Here, asymmetric vesicles are prepared by exposing unilamellar vesicles to a solution of cyclodextrin derivative loaded with the desired lipid species. Only the outer leaflet of the vesicle bilayer can directly exchange lipids and thus be enriched with the lipid previously in complex with the CD. This approach is especially appealing since it allows for the generation of asymmetry with a variety of lipid compositions and, as shown recently, incorporation of a highly controlled level of cholesterol in the asymmetric vesicle.[6]

Rather than using exchange, lipid vesicles with highly controlled asymmetry can be assembled in a step-by-step fashion. Microfluidic technologies, which are capable of efficiently generating monodisperse droplets, have been applied in the production of asymmetric vesicles using a layer-by-layer assembly approach.[11] In another example involving such an assembly strategy, Weitz and co-workers[8] have reported a water-in-oil emulsion-based method wherein the inner monolayer is first formed via the emulsification of water droplets in oil containing the first amphiphile of interest. The outer leaflet is subsequently generated by forcing the emulsified water droplets through a second oil-water interface containing the second amphiphile using centrifugation. This method allows for encapsulation efficiencies of almost 100%, addressing another common challenge when making synthetic vesicles.[86] This strategy has been adopted[87] to asymmetrically distribute PS at the outer leaflet of the vesicle to resemble apoptotic bodies and phosphatidic acid (PA) at the inner layer to enhance innate antimycobacterial activity in phagocytes while limiting the inflammatory response. These asymmetric apoptotic body-like vesicles provide a promising immunotherapeutic platform for *Mycobacterium tuberculosis*.

In biological membranes, the final organization of proteins in lipid bilayers is mainly governed by three parameters – protein characteristics, the aqueous extra-membrane environment and lipid composition.[66, 88] The charge and specific hydrophilic and hydrophobic domains of the lipids directly influence the processes of insertion, folding and topology of proteins in the membrane. Nevertheless, the principles and detailed mechanisms of lipid-dependent assembly and organization of membrane proteins are

still elusive. Reconstitution of membrane proteins into artificial vesicles is thus used to elucidate the mechanisms by which membrane proteins interact with lipids in native membranes.[89, 90] However, there are technical challenges regarding effective control over protein orientation within a vesicle bilayer. Proteins should not be randomly distributed and should be integrated in a desired orientation. Inspired by the earlier work on the formation of asymmetric vesicles using water-in-oil emulsion-based method, Oiki and co-workers[90] have successfully reconstituted the membrane potassium channel KcsA with either an outside-out or inside-out orientation in giant unilamellar vesicles (**Figure 4**). The lipid composition of the inner and outer leaflets was varied in a systematic manner and shown to influence protein insertion capability and rate, as well as protein channel function. The addition of KcsA in either an intravesicular or extravesicular solution dictated location of the pH-sensitive cytoplasmic domain (CPD) inside or outside of the vesicle. Indeed, the mechanism underlying the direct KcsA insertion was shown to be governed by the hydrophilic CPD, which hardly traversed the hydrophobic core of the bilayer, and thus was retained in the aqueous phase to which the KcsA was added.

By using *in vitro* reconstitution vesicle systems, researchers are unveiling new insights into the dynamic lipid-protein molecular interactions that can have important implications to the development of therapeutic approaches for disorders in which lipids play an important role. Notwithstanding, the versatility of the strategies reported, which enable asymmetric tunability of the vesicle's molecular properties, are still to be further explored and developed to aid in the challenging undertaking of understanding the role of lipid asymmetry in membrane structure and function. Synthetic asymmetric lipid-based vesicles also suffer from time-induced loss of asymmetry due to transverse diffusion (flip-flop). The current lifetime is in the order of hours to days, which is strongly dependent on the lipid structure[91], and thus strategies to prepare more stable artificial asymmetric vesicles will be desired.

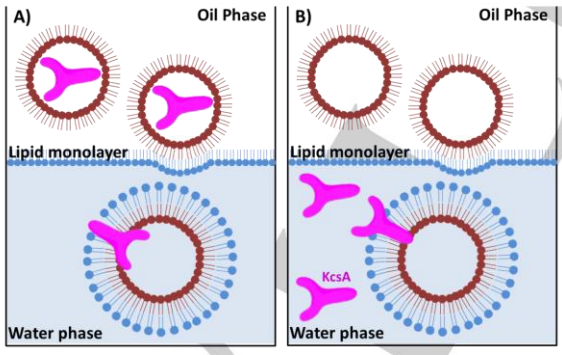


Figure 4. Schematic representation of the water-in-oil emulsion-based method used to obtain vesicles containing KcsA in either orientation. KcsA insertion into vesicles from (A) the inside and (B) outside, where the lipids of the outer leaflet are chemically different from those of the inner leaflet.

4. Membrane binding and downstream events

EVs bind with the plasma membrane of target cells by ligand-receptor interactions, fusion or internalization.[92] The ligand-receptor binding can be determined by several adhesion proteins present in EVs, including integrins, intracellular adhesion molecule 1 (ICAM1), tetraspanins and galectins, which have been shown to interact with membrane receptors and induce adhesion-dependent intracellular signaling events in the target cells.[93]

It is well recognized that rafts play an important role in signal transduction by containing different signaling proteins which may cluster or fuse upon agonist stimulation, resulting in downstream signaling. Transferrin receptors have been shown to be a raft component in exosomes that upon stimulation by crosslinking induce downstream signaling pathways of target cells by triggering mitogen-activated protein kinase (MAPK) activation.[94] Receptor-ligand interactions mediated by exosomes on target cells have been also shown to suppress key tumor cell recognition pathways.[95]

In other cases, however, binding is followed by the direct fusion of the vesicle with the plasma membrane of the target cell. This fusion results in the integration of the vesicle membrane proteins into the membrane of target cells and the release of their contents into the cytoplasm to activate downstream events in target cells. The mechanism of EV-cell fusion is not well understood, but may involve integrins for adhesion and tetraspanin-enriched microdomains to facilitate exosome fusion.[96, 97]

EVs can also be internalized via receptor-mediated endocytosis by either the clathrin-dependent[98] or the lipid raft-dependent endocytic pathways.[99] Endocytic uptake can be followed by fusion with the endosomal membrane. Phagocytosis is also a means of exosome internalization. Because of an enrichment of PS in the outer layer of EVs, multiple PS binding proteins on target cells, including two members of the Tim (T-cell immunoglobulin-containing and mucin-domain-containing molecule) family transmembrane proteins Tim1 and Tim4) can bind exosomes and trigger phagocytic uptake of EVs.[100]

In synthetic vesicles, membrane binding and downstream events have been facilitated by supramolecular chemistry and its molecular recognition principles. Molecular recognition of small and large molecules at the surface of vesicles has been accomplished either by selective metal-ligand coordination, hydrogen bonding and host-guest interactions.[101] In particular, host-guest interactions have been highly successful in reorganizing and assembling molecular constituents into the vesicle membrane bilayer. For instance, unilamellar vesicles comprising amphiphilic α- and β-CD have been formed and exposed to a divalent adamantyl guest to mimic receptor clustering through multivalent interactions.[102] The selective affinity of the adamantyl moiety for the β-CD and the fluidity of the bilayer have led to the binding of the two adamantyl moieties to two β-CD in the vesicle, thus enabling β-CD clustering, in a similar fashion to clustering in clathrin-mediated endocytosis. Multivalent interactions have been also demonstrated to occur on vesicles formed using amphiphilic cucurbit[6]uril (CB[6]) derivatives.[103] Exposure of the vesicles to α-mannose-substituted spermidine led to vesicles functionalized with α-mannose moieties, which bound specifically to the lectin concanavalin A (Con A) in a multivalent manner.

Through a variety of functionalizations, researchers have also developed strategies to transform binding events occurring at the vesicle interface into a response (e.g. a change in fluorescence signal). These efforts, with potential sensing applications, typically rely on co-embedding the receptors and the fluorescent reporters in the vesicle bilayer.[104, 105, 106] Upon binding, receptor sites are spatially re-organized, thus affecting the optical properties of co-embedded reporters and triggering a change in the

10.1002/anie.201607825

vesicle emission properties. This strategy has been employed to create vesicular aptasensors for the detection of thrombin[106] and adenosine.[107] Molecular recognition and signal transduction processes at the surface of vesicles have also been harnessed to control enzymatic activity.[108]

In addition to transducing signals at the outer surface of the vesicles, researchers also embarked on studies aimed at mimicking cellular aspects of signal transduction across vesicle lipid bilayers. External small-molecule triggers, which passively diffuse into the vesicle interior, have been shown to reversibly control dynamic protein–ligand interactions in giant vesicles.[109] Through interior enzymatic conversion of two different small-molecule substrates, increase or decrease in the interior pH occurs that allows modulation of the pH-sensitive interaction between a Ni-NTA ligand on the vesicle membrane and a His-tagged protein in the lumen.

In line with other mechanisms of transduction in cell membranes, which require changes in the organization of membrane components and receptor clustering processes, vesicles have been devised to transmit binding information across their lipid bilayers. Examples include the use of two different unsymmetrical membrane-spanning transmitter units that in the presence of an external signal molecule can dimerize, bringing internal signaling groups into close proximity. In turn, this has led to the possibility of stimulating internal FRET effects,[110] inducing metal (i.e. Cu^{2+}) complexation[111] and intramolecular reactions at the inner bilayer surface.[112, 113] Regarding the latter, Schrader and co-workers[113] have reported lithocholic acid-based transmembrane blocks functionalized with bisphosphonate dianions on one side for oligoamine-recognition and a pair of thiol nucleophile and pyridine disulfide substrate for intravesicle S_N2 displacement on the other side (**Figure 5**). The induced proximity of two transmembrane molecules units to one another inside the fluidic membrane facilitated the S_N2 attack of the thiol onto the pyridine disulfide, resulting in the instantaneous release of thiopyridine.

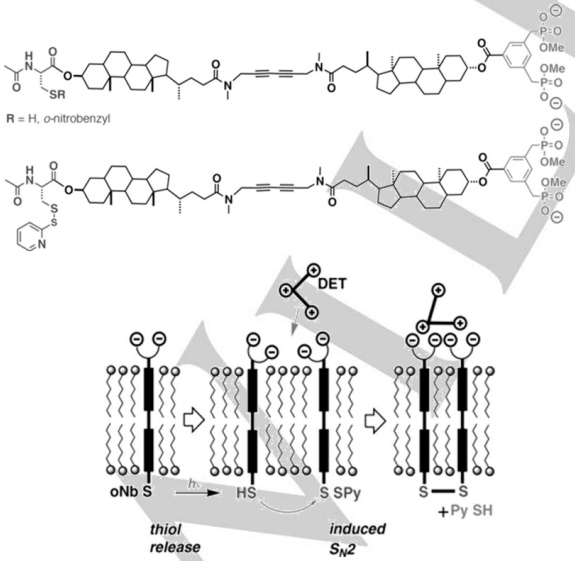


Figure 5. Chemical structure of the membrane spanning-molecules and chemical scheme showing the sensing and signaling reaction in the lipid bilayer of vesicles.[113] Exposure of 200 nm vesicles to diethylenetriamine (DET) led to the formation of a double chelate complex with two bisphosphate units in the bilayer, leading to the release of thiopyridine.

These systems still face challenges related to the correct orientation of the transmembrane so that all receptor heads and reactive sites point away from the outer and inner bilayer surface, respectively. Furthermore, systems should be devised that lead only to the formation of heterodimers and not to the assembly of two homodimeric entities. In spite of the vast importance of transmembrane signaling in nature, there is a paucity of published work in the design and assembly of artificial signal transduction vesicles. Consequently, future research in this area should be undertaken in order to build vesicle models that more closely reflect the complexity of signal transduction processes at natural cell membranes.

Dynamic molecular interactions and rearrangement within vesicle bilayers have been also of considerable interest to understand the fundamental physical rules governing vesicle fusion Membrane fusion is a ubiquitous and critical event in all living organisms, being the basis of many transmembrane transport processes, such as synaptic neurotransmission, endocytosis and exocytosis. The development of vesicle constructs that are able to control and direct the fusion of membranes will not only contribute to a better understanding of how fusion is naturally occurring, but will also open up new opportunities in the fields of materials science and the design of drug and gene transfection delivery tools.[114, 115] *In vivo* membrane fusion is a highly controlled process. To mimic this process Hook and co-workers[116] exploited the hybridization of membrane-anchored DNA strands to selectively fuse lipid vesicles (**Figure 6**). To this end, DNA strands, which were partially duplexed, were modified with cholesterol to ensure spontaneous incorporation into the lipid bilayer of the vesicles. The orientation of the DNA strands with respect to the cholesterol anchor was designed such that hybridization occurred in a zipper-like fashion, only bringing vesicles with complementary DNA sequences into close proximity. This strategy triggered the fusion of the vesicles in a manner that resembles the mode of action of the complex SNARE fusion proteins during native fusion.[56]

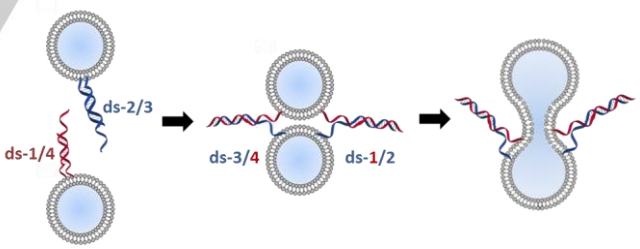


Figure 6. Vesicle fusion mediated by DNA hybridization. Initially, vesicles were modified with the cholesterol-terminated DNA strands ds-1/4 and ds-2/3 (left side). As ds-1/4 and ds-2/3 encounter each other, they hybridized in a zipper-like fashion, thus forming blunt-ended duplexes with 27 base pairs (ds-1/2) and 12 base pairs (ds-3/4) (middle). In this geometry, the bilayers were brought in contact with each other, leading to opening of the fusion pore (right side).[116]

Fusion of vesicles by molecular recognition has been also achieved using metal coordination,[117, 118] host-guest interactions,[119] and coiled coil peptide interactions.[120] For example, Richard and co-workers[118] demonstrated the fusion of vesicles by the selective recognition between specific metal ions and vesicles bearing bipyridine ligands. Other model systems for membrane fusion have been constructed based on the selective recognition between boronic acids and *cys*-diols[121] and com-

plementary coiled-coil-forming peptides.[120] In the latter, one of the peptides was non-covalently decorated in cyclodextrin-based vesicles while the complementary was incorporated into phospholipid vesicles using a cholesterol anchor. These investigations provide new insights into the complex chain of events of protein-induced membrane fusion, highlighting the importance of vesicle proximity and force transmission in membrane fusion. Fusion between vesicles opens up the possibility to add and mix the contents of different vesicles as will be discussed in the following sections.

5. Vesicle compartmentalization

One of the key roles of lipidic membranes in natural systems is to separate vital components into different compartments where they can be stored and protected (e.g. genetic information within the nucleus); trafficked within (e.g. endosomes) and outside (e.g. exosomes) cellular environments; or where functionality can be centralized (e.g. protein manufacture within the endoplasmic reticulum). This exquisite level of organization has been an inspiration for researchers to develop novel vesicle within vesicle systems that can find applications in drug delivery, sensing or as microreactors.[122, 123, 124]

In natural systems, compartmentalization is a tightly regulated process where the creation of new compartments such as endosomes, lysosomes or multivesicular bodies is controlled by membrane receptors, signaling networks and structural proteins. For instance, uptake of nutrients, toxins and pathogens into endosomes and lysosomes is regulated by the endocytosis pathway.[125, 126] Binding at the cell surface dictates which of the alternate pathways (e.g. calthrin vs caveolin-mediated) will be employed. This binding then results in structural changes in the membrane and cellular components, including changes to lipid composition and recruitment of structural proteins, which then lead to membrane budding and the formation of new vesicles within the cell.[127, 128]

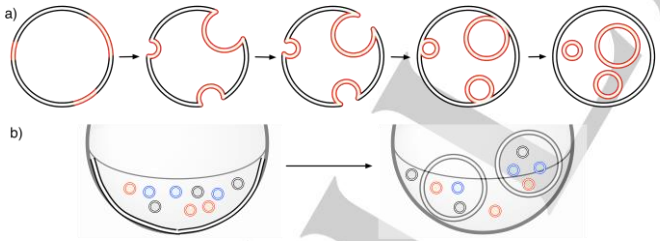


Figure 7. Strategies commonly employed to prepare compartmentalized vesicles. A) Compartments are generated by invagination of vesicles from heterogeneities generated in the membrane. B) SUV are encapsulated inside a newly formed vesicle. The latter process if often facilitated by membrane binding or the use of microfluidics.

While reproducing all of these features in a synthetic system is currently out of reach, researchers have often mimicked some of this machinery in order to drive compartmentalization in synthetic vesicles (**Figure 7**). For example, heterogeneities in membrane composition, or changes in the chemical environment at the membrane interface, can be exploited to trigger the deformation of vesicles and the formation of novel compartments. For instance, the membrane of GUVs is normally destabilized under stress (e.g. osmotic shock or treatment with a surfactant) forming asymmetric vesicles.[129] Budding of the surface can then occur and lead to invagination of smaller vesicles, in a process analogous to the formation of ILVs in natural systems. Takagi and co-workers[130] demonstrated that the presence of raft domains in these GUVs controls invagination, with the size and distribution of these domains having an important effect on the overall process. When raft domains were “evenly” distributed, they would curve inward to eventually become an invaginated vesicle. The size of the enclosed vesicles depended on the raft domain size, with smaller domains budding first, followed by mid-sized domains. Large raft domains however produced monodisperse endocytic vesicles with multiple vesicles budding from the boundary. Okomura and co-workers have recently used a similar strategy for the compartmentalization of GUV prepared by electroformation.[131]

As a result of this process, synthetic invaginated vesicles have very similar membrane compositions than the GUVs they originated from. However, this can be a limiting factor that prevents researchers from exploiting differences in membrane composition in these compartments. In this regard, Vogel and co-workers demonstrated that the use of different lipid formulations in each compartment, with different melting points (dipalmitoyl ~ 41 ºC vs dimyristoyl ~ 23 ºC vs dioleoyl ~ -18 ºC) enables sequential release of the contents within the compartmentalized vesicle, something that can be of benefit in the application of these synthetic vesicles (**Figure 8**).[132, 133] In this case, vesicles were prepared by a film-hydration protocol that enabled co-encapsulation of small unilamellar vesicles (SUVs) with different membrane composition.

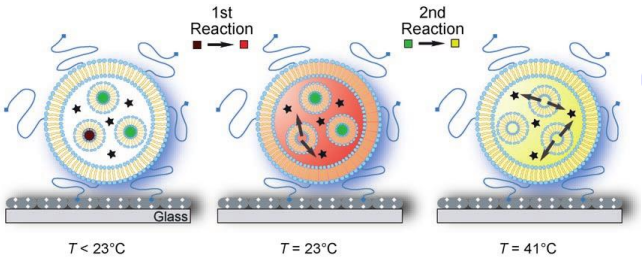


Figure 8. Schematic representation of consecutive enzymatic reactions in compartmentalized vesicles. The external nanoreactor surface carries biotin for immobilization on a neutravidin-coated glass slide. An increase of temperature triggers the release of the substrates in two distinct, consecutive steps at the two corresponding phase-transition temperatures, ~ 23 ºC and then ~ 41 ºC. After release from the SUVs, the substrates remain confined in the nanoreactor, where they are converted by the enzyme to their particular fluorescent products.[132]

Alternatively, researchers have employed molecular recognition between complementary bilayers to control compartmentalization. This approach, which mimics the binding events that mediate vesicle formation in natural systems and how these vesicles are then trafficked, was early illustrated by the group of Zasadzinski with the encapsulation of SUVs inside large unilammelar vesicles (LUVs) formed upon unrolling of phosphatidylserine cochleate cylinders (**Figure 9**).[134] SUVs and cochleate cylinders were functionalized with biotin to enable compartmentalization by ligand-receptor binding at the SUV-LUV interface. Addition of sub-stoichiometric streptavidin facilitated SUV aggregation, while ensuring that enough biotin moieties were still

available. Cochleate cylinders on the other hand were saturated with streptavidin, allowing that the biotin-functionalized SUV-clusters and streptavidin-functionalized cochleates interacted and formed compartmentalized vesicles, coined vesosomes by the authors. At the time, this strategy presented several challenges such as poor encapsulation efficiency (5-15% of the SUV encapsulated) and purity, with a mixture of LUVs, vesosomes, free aggregates and free vesicles being formed. Some of these challenges have now been addressed using alternative encapsulation protocols,[135] or purification steps (i.e. centrifugation, sedimentation).[136]

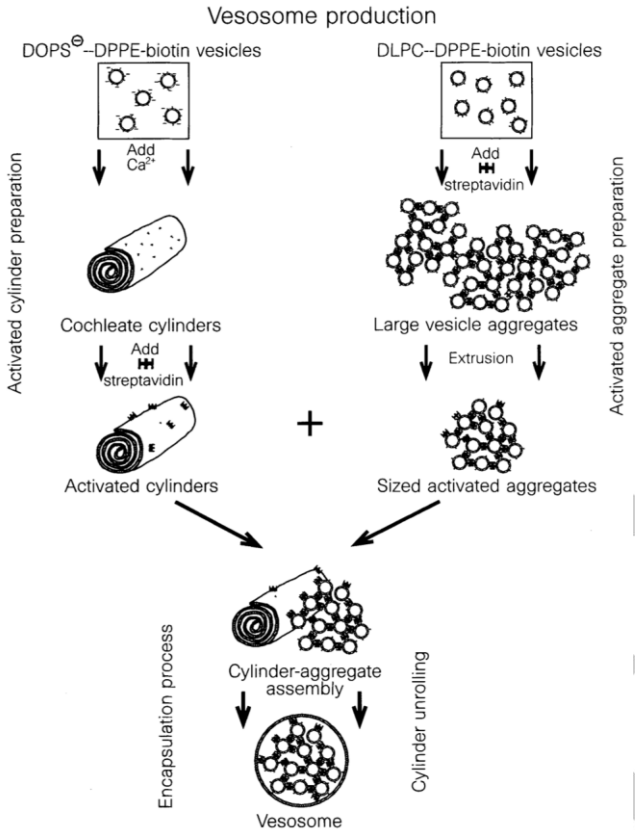


Figure 9. Schematic representation of the synthesis of compartmentalized vesicles reported by Zasadzinski and co-workers.[134]

Preserving the integrity of vesicles, while a secondary membrane is being formed, is a key aspect in compartmentalization that is normally achieved by a careful choice of membrane composition. All in all, fusion of the formed compartments must be avoided. In this regard, Paleos and co-workers have carefully evaluated the effect of membrane composition and particle size over vesicle fusion and compartmentalization.[123, 137] Taking advantage of the supramolecular interaction between guanidinium and phosphate moieties to trigger vesicle binding, the authors demonstrated that several events can occur upon binding, namely adhesion, fusion (**Figure 10**, top) and/or compartmentalization (**Figure 10**, bottom). For instance, addition of cholesterol increased the membrane fluidity and facilitated interfacial recognition and fusion. Interestingly, vesicle adhesion and fusion occurred through mixing of the lipids but without leakage of the vesicle content. It was proposed that adhesion of LUVs to GUVs is the key step in the formation of compartmentalized vesicles, where LUVs are engulfed by the GUV, in a process similar to cell endocytosis (**Figure 10**).

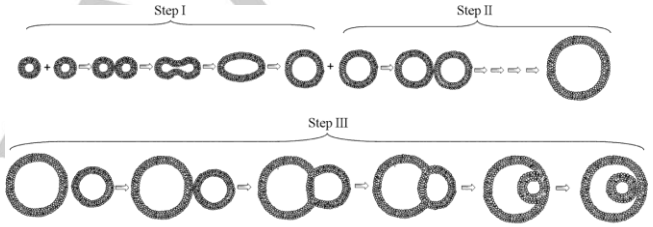


Figure 10. Mechanistic scheme for the formation of multicompartment vesicles Step I: Fusion, Step II: Growth and Step II: Engulfment.[123]

Parallel to this progress in vesicle synthesis and design, recent advances in automation and microfluidics[138, 139] are allowing researchers to design compartmentalized vesicular systems with narrow size distributions and excellent control over the number of compartments.[7] Here, monodisperse GUVs were prepared first using a microfluidic double emulsion technique.[140] These GUVs were then subjected to a second microfluidic double emulsion process to yield the desired compartmentalized polymersomes. The amount of vesicles encapsulated (up to 3) was controlled by the diameter of the injecting capillary tube and GUV containing different dyes could be co-encapsulated using this technique (**Figure 11**, Left).

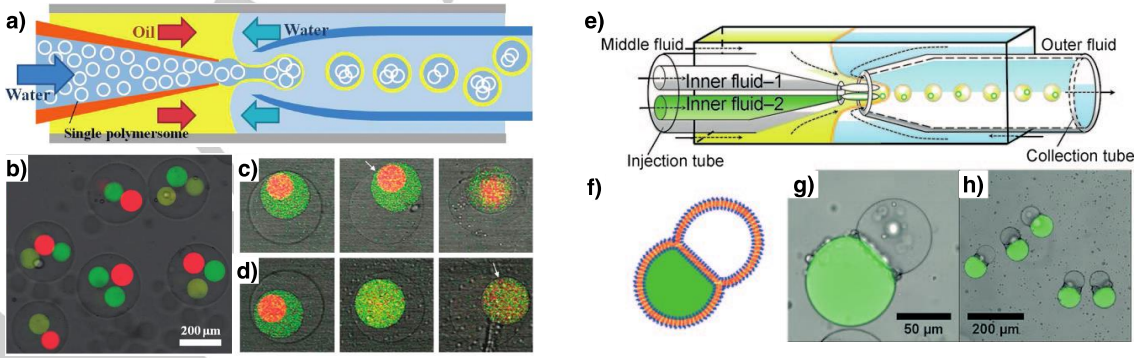


Figure 11. A) Schematic illustration of the microfluidic device to prepare polymersomes containing several inner polymersomes and B-D) confocal microscope images.[7] E) Schematic illustration of the microfluidic device to prepare asymmetric compartmentalized polymersome and F-H) confocal microscope images.[10]

Similarly, our current understanding of membrane chemistry, and vesicle binding and fusion, is allowing researchers to produce non-spherical compartmentalized vesicles with excellent precision.[10] In this case, GUVs were encapsulated within an oil droplet and the organic phase allowed to evaporate to induce vesicle adhesion, fusion of the membranes and the formation of compartmentalized vesicles. Again, the use of microfluidics provided the precise control needed and vesicles with up to 10 compartments could be prepared. Moreover, the use of two inner fluids in the injector tube facilitated the formation of anisotropic/asymmetric vesicles that encapsulated different solutes within different compartments (**Figure 11**, right).

These examples highlight how researchers are now capitalising on our understanding of lipid chemistry and physics, to design complex compartmentalized vesicular systems. These compartmentalized vesicles will not only improve the release kinetics of drugs,[139, 141] but will allow us to confine complex chemical transformations to vesicular systems, in an attempt to improve current chemical process, but also imitate some of the features observed in natural cells. Further discussion of these aspects is provided in the next sections.

6. Chemical nanoreactors

While there are no reports of the synthetic capability in EVs, researchers have been attracted for decades by the possibility of carrying out chemical transformations within the confined space of a vesicle.[142] Like in cellular systems, reaction rate is often increased due to concentration effects inside the bilayer, ion distribution at the vesicular interface or enhanced reactivity inside the vesicle.[143] More importantly, location of substrates, catalysts and/or products is restricted to the vesicle, a feature that nature constantly exploits to either protect valuable products (e.g. nucleic acids in the nucleus) or confine harsh conditions in localized compartments (e.g. enzymatic degradation in the lysosomes). In a similar fashion, researchers have now encapsulated enzymes and nucleic acids in synthetic vesicles where they can be protected from degradation and quite often stabilized due to macromolecular crowding[144] or stabilization at hydrophobic regions.[145] The potential of imitating natural systems using synthetic vesicles was first reported by Nolte and co-workers in 1986.[146] In this system, the oxidation of simple alkenes was catalyzed by a synthetic model of cytochrome P-450. To function, the synthetic model required a membrane bound manganese porphyrin coupled to an axial ligand (N-methylimidazole), an electron donor (colloidal Pt) and an electron carrier (methylene blue), thus mimicking all of the features of the natural system. This biomimetic nanoreactor was able to reduce Mn^{III} to Mn^{II} and produce the desired epoxides only in the presence of all of these components.

The trade-off between vesicle stability and membrane permeability is possibly the biggest challenge when designing vesicular reactors. Vesicle membrane has to be permeable to the reagents and products, while containing the catalytic species. In cellular systems, permeability is dictated by the composition of the membrane – that controls the passive diffusion of “small” solutes, and the presence of channels and pores – that regulate the, often active, transport of larger solutes.[147] Permeability in synthetic systems though is often dictated only by the choice of membrane components, which control the physical properties of the membrane, including the viscosity and crystallinity of the bilayer.[148, 149, 150, 151] Like in natural membranes, cholesterol is often inserted in lipidic membranes to increase their toughness while maintaining their elasticity, so that stable membranes with “controlled” permeability can be prepared. However, increasing membrane permeability can eventually result in higher vesicle instability, as illustrated by the destabilization of model lipidic membranes at “high” content of cholesterol.[152, 153] The stability of synthetic vesicles is further affected when used in complex media, such as that found in biological applications. Absorption of proteins and other biomolecules can result in lysis of the membrane and opsonization and uptake by macrophages for in-vivo applications.[154] Coating vesicles with large “hydrophilic” polymers such as poly(ethylene glycol) (PEG) can increase the steric hindrance around the vesicles, thus minimising adsorption of biomolecules and increasing the stability (and lifetime) of the vesicles.[154, 155, 156] However, this steric hindrance also compromises the diffusion of solutes across the membrane. Overall, long-term stability of the vesicle is highly desired when considering applications and it is not surprising that most of the research in this area has focused on the application of polymersomes in the synthesis of vesicular reactors.[142, 157, 158, 159] Polymeric amphiphiles have bigger dimensions than lipids and polymersome membranes can be up to 5 times thicker than lipidic ones.[160, 161] This increased thickness and the tendency of polymer chains to entangle make polymersomes less likely to break apart. However, the diffusion of solutes through the polymersome membrane is compromised and makes most polymersomes impermeable to small molecules, with only a handful of polymersomes showing intrinsic permeability.[162, 163, 164]

Researchers have exploited several strategies to improve the permeability of polymersomes.[165] One of the most effective approaches is exploiting natural membrane channels, which can be reconstituted in synthetic vesicles to produce pores of well-defined sizes. This technology was first introduced by Meier and co-workers using poly(2-methyloxazoline)-*b*-poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline) PMOXA-PDMS-PMOXA polymersomes that were permeabilized using a bacterial porin (OmpF).[166] These polymersomes were permeable to small molecules such as ampicillin and encapsulation of a β-lactamase enabled the transformation of ampicillin to ampicillinoic acid within the aqueous lumen. In the absence of OmpF, no reaction was observed while encapsulation had a minor effect on the kinetics of the transformation. This strategy has been widely used and there are now examples of nanoreactors, which incorporate OmpF and other protein channels,[158, 159] to produce permeable polymersomes with applications in drug delivery,[167] as antioxidants[168] or in enzyme replacement therapy.[169]

In recent years, not only single components but whole machineries have been encapsulated within synthetic vesicles, in an attempt to engineer nanoreactors that imitate some of the characteristics of natural cells. For example, the group of Libchaber was able to encapsulate an *Escherichia coli* extract that contained all of the components needed for the transcription–translation of encapsulated plasmids, including amino-acids, ribonucleotides, ribosomes, tRNA and T7 RNA polymerase.[170] The authors relied on a water-in-oil emulsion-based preparation to maximize the amount of extract encapsulated. A α-hemolysin-eGFP fusion protein was then engineered, so that upon expression inside the vesicle, α-hemolysin would assemble into the membrane-active heptamer forming pores of 1.4 nm in diameter.

Efficient pore production during the first hours of the experiment ensured that nutrients were allowed to diffuse inside the vesicles to avoid exhaustion of the amino acids and nucleotides initially encapsulated, so that protein production could be maintained for more than four days.

While a lot of research has been focused in "borrowing" components from natural systems, having access to the arsenal provided by synthetic chemistry opens up the path to alternative strategies for membrane permeability. For instance, incorporating stimuli-responsive materials can facilitate transport in vesicles and nanoreactors, because changes in external conditions can trigger changes in their solubility and facilitate the diffusion of molecules across the membrane.[157] For example, van Hest and co-workers exploited this principle for the synthesis of glucose-responsive nanoreactors with controllable permeability.[171] In this case, poly(ethylene glycol)-b-poly(styrene boronic acid) (PEG-b-PSBA) was blended with poly(ethylene glycol)-b-poly(styrene). The PEG-b-PSBA was selectively dissolved in the presence of glucose (or at high pH) creating pores in the membrane of the nanoreactors. The activity of CalB encapsulated in the lumen of these polymersomes was shown to increase after removal of the PEG-b-PSBA blocks. No esterase activity was observed in the filtrate of purified "opened" nanoreactors, suggesting that CalB was retained within the polymeric vesicles. Other stimuli such as pH,[172, 173] CO_2[174] and UV[175] have been also exploited by other groups to increase the permeability of nanoreactors.

In natural systems, location of enzymes is not restricted to the aqueous lumen and chemical transformations occur both at the membrane, inside the vesicular system and at the interface. This has inspired researchers to develop nanoreactors that can not only encapsulate active enzymes in the hydrophobic membrane,[176, 177] but implement cascade reactions by colocalization of more than one enzyme within the same vesicular system. Enzymes can thus be located at the membrane surface, within the membrane or inside the vesicle to facilitate the sequential reaction of the products formed. A catalytic system composed of 3 enzymes - CalB, horseradish peroxidase (HRP) and glucose oxidase (GOX) – was the first demonstrating the potential of this approach (**Figure 12A**).[178, 179] HRP was encapsulated in the vesicle membrane following co-liophilization with the polymeric amphiphile, dissolution in THF of this polymer-enzyme mixture and addition to an aqueous solution. GOX dissolved in this second aqueous solution was selectively encapsulated in the aqueous lumen. CalB in solution (outside of the polymersomes) hydrolyzed 1,2,3,4-tetra-O-acetyl-b-glucopyranose to form glucose that was then oxidized by the GOX encapsulated in the aqueous lumen forming hydrogen peroxide as a by-product. This hydrogen peroxide was finally used by the HRP in the membrane to oxidize 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS), giving the coloured compound that allowed monitoring of the whole cascade. These catalytic systems normally result in an increase in the enzyme half-life, and in this particular case, the reported cascade was able to work for over 24 h, three times longer than when the enzymes were colocated free in solution. Alternatively, the same authors have demonstrated that CalB could be encapsulated in the membrane while HRP was covalently attached to the surface of the polymersome, with similar catalytic results (**Figure 12B**).[179]

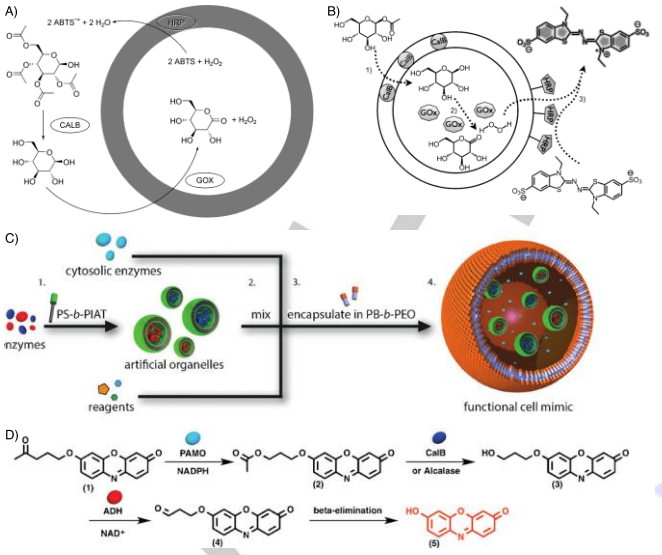


Figure 12. A) and B) Cascade nanoreactors exploiting the intrinsic potential of vesicles to segregate cargo.[178, 179] C) and D) Cascade nanoreactor based on additional vesicular compartments.[180]

Recent developments in the synthesis of compartmentalized vesicles are allowing researchers to prepare vesicle within vesicle nanoreactors that can also encapsulate cascade reactions.[180] In this case, SUVs formed from poly(styrene)-block-[3-(isocyano-L-alanyl-amino-ethyl)-thiophene] (PSt-PIAT) were used to encapsulate two sets of enzymes: CalB or alcohol dehydrogenase (ADH). These enzyme-loaded polymersomes were then co-encapsulated within polybutadiene-b-poly(ethylene oxide) GUVs together with a fusion protein of phenylacetone monooxygenase (PAMO) and nicotinamide adenine dinucleotide phosphate (NADPH), and pro-fluorescent 7-((4-oxopentyl)oxy)-3H-phenoxazin-3-one (**Figure 12**C). Encapsulation protected the enzymes from degradation by proteases while having a minor effect over the enzymatic activity. This system mimics compartmentalization within eukaryotic cells and highlights the potential of vesicles in synthetic biology and biotechnology. Other cascade reactors have been reported,[181, 182] some of which have applications for cofactor regeneration,[183] as antioxidants[180, 184] or as antimicrobials.[185] This ability to carry complex chemical transformations inside vesicles has encouraged researchers to use nanoreactors as artificial organelles that reproduce cellular behavio**u**r, restore function to damaged cells or even improve their performance.[159, 186, 187] The incorporation of nanoreactors into cells was first demonstrated by Hunziker and co-workers with the internalization of trypsin loaded polymer vesicles by THP-1 macrophages.[188] PolyG oligonucleotides on the surface of PMOXA-PDMS-PMOXA polymersomes promoted recognition by macrophages and internalization, and trypsin-loaded polymersomes remained active once internalized.

Further developments in this area have resulted in the implementation of cascade reactions within artificial organelles. Towards this end, Moore and co-workers reported a liposomal reactor that incorporated both the F_0F_1-ATP synthase motor protein and the photoinduced proton pump carotene-porphyrin-naphtoquinone (C-F-Q) in its membrane.[189] Liposomes were prepared by the Biobeads method,[190] which ensured that F_0F_1-ATP synthase was reconstituted with the right orientation. An

inward flow of protons was established upon irradiation of the C-F-Q triad, protons that were required to activate the F_0F_1-ATP synthase motor. Overall, the organelle was able to produce ATP, the key intermediate in the intracellular transfer of energy (**Figure 13**A). Ensuring that the protein channel has the correct orientation is key in this synthesis, and formulations that result in an statistical orientation of this channel can have a detrimental effect in the performance of these artificial organelles.[191]

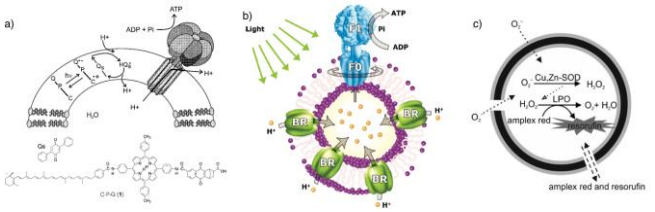


Figure 13. Schematic of artificial organelles incorporating F_0F_1-ATP synthase and (A) C-P-Q proton pump [189] or (B) bacteriorhodopsin,[191] or (C) a cascade reaction within the vesicle lumen.[168]

Cascade reactions in synthetic organelles can be also implemented by co-encapsulation of enzymes in the aqueous lumen, an option that eliminates the drawbacks associated with the lack of control over the orientation of membrane channels. This strategy has been demonstrated with the internalization by THP-1 cells of polymersomes loaded with superoxide dismutase (SOD) and lactoperoxidase (LPO).[168] These nanoreactors retained their activity within the cells and were able to work as antioxidants, allowing the in situ detection and detoxification of intracellular reactive oxygen species (**Figure 13C**). Interestingly, these polymersomes carried the channel protein OmpF to facilitate the diffusion of LPO substrates/products across the membrane and increase the activity of these nanoreactors.

Altogether, the increasing control over the positional assembly of enzymes and catalysts, and the ability to facilitate diffusion of reagents and products across membranes, is enabling researchers to implement complex multistep chemical transformations within vesicular systems. This is allowing researchers to now close the cycle and feedback to biological research, with the development of artificial nanoreactors that can impact biological systems. These sophisticated nanoreactors should not only be able to reproduce and repair cellular behaviour, but eventually imitate all of the characteristics of natural systems.

7. Artificial Minimal Cells

The development of artificial minimal cells is possibly the ultimate challenge in the synthesis and application of vesicular systems. Natural cells incorporate all of the features described above, including membrane asymmetry, functional compartments and the ability to carry complex chemical transformations. Not surprisingly, implementing most (if not all) of these characteristics in a single synthetic system has fascinated researchers for decades.[192, 193, 194] In particular, research in this area has focused in developing vesicular systems that go beyond the simple imitation of biological processes,[195] but can grow and self-replicate.[194]

This ability of synthetic liposomes to self-replicate was first reported by Luisi's group, using an enzymatic cascade to facilitate the synthesis of phosphatidylcholine derivatives within soybean phosphatidylcholine liposomes.[196] A further level of complexity was then reported, where the encapsulation of a phosphorylase (PNPase) within oleic acid/oleate vesicles enabled the polymerization of ADP within the vesicles - a process that modelled a cellular metabolism.[197] Autocatalytic hydrolysis of exogenous oleic anhydride in the aqueous lumen facilitated growth and self-reproduction of these vesicles,[198] constituting the first fully synthetic system capable of containing a metabolism while at the same time growing and self-reproducing.

A common limitation when developing synthetic cells is to control the permeability of the membrane to facilitate diffusion of the "nutrients" while containing the machinery needed for the replication/templation of the genetic information. Nature uses a sophisticated network of membrane channels, organelles and compartments, and tightly regulates trafficking of these components to facilitate transport of "nutrients" and products. Researchers however do not have access to this degree of control and have relied alternatively on optimising membrane composition and properties. For instance, Szostak and co-workers postulated that small fatty acids and their derivatives could increase the permeability of protocells.[199] In their approach, myristoleic acid and decanoic acid vesicles showed significantly higher permeability than phospholipidic vesicles to a range of model substrates, including ribose and nucleotides. Permeability was increased when "defects" were introduced in the membrane, blending fatty acids with other lipids (e.g. glycerol monoester of decanoic acid). However, charged nucleotides were not able to diffuse through the membrane on their own, limiting the application of these vesicles. Conversely, imidazole-activated nucleotides were able to diffuse through these fatty acid vesicles, enabling the non-enzymatic replication of a DNA oligonucleotide inside the vesicles. This strategy can potentially be coupled with the controlled growth and replication of fatty acid vesicles, when fed with fatty acid micelles,[200] for the development of prebiotic models of cells.

Ensuring that cellular content is equally distributed between the daughter cells is another challenge when developing synthetic cells. Again, in nature this process is tightly regulated by a complex network of signals and scaffolds that can distribute cellular components into the dividing cells. In synthetic systems, as the system becomes more sophisticated, it is less likely that the replicating cells will maintain all of the original components and thus the replicating cellular systems quickly become non-functional. Sugawara and co-workers addressed this limitation linking the process of self-replication of the information with the self-reproduction of the vesicle compartment.[201] In their approach, DNA was amplified inside a model vesicle using PCR (**Figure 14**). Cleavage at the membrane interface of an imine bond in a cationic membrane precursor triggered the formation of membrane components, vesicle growth and self-reproduction. More importantly, self-reproduction increased the cationic content of the growing vesicles, so that amplified DNA was able to associate with the growing membrane. Accumulation of cationic amphiphiles around amplified DNA eventually created an imbalance in the membrane composition that lead to pre-organization and budding/deformation of these protocells. While this strategy is not perfect, and there are still issues such as maintaining the concentration of non-reproducing neutral and anionic lipids needed to ensure long term stability of the formed synthetic cells, it highlights the potential of mimicking natural systems in their

ability to couple the replication of genetic information with structural and morphological changes to trigger cell division.[202]

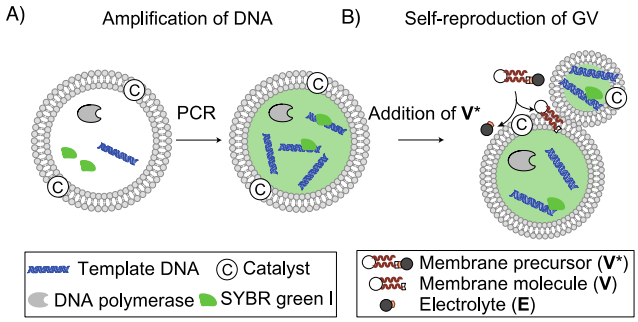


Figure 14. Schematic representation of the chemical link between (A) amplification of DNA and (B) self-reproduction of GVs.[201]

8. Summary and Outlook

In this review, we have highlighted recent progress in the understanding of natural vesicles and current efforts to prepare increasingly sophisticated synthetic vesicles. In recent years, our knowledge of the origin and different roles of natural vesicles has constantly increased. In the same fashion, we have improved our understanding of membrane chemistry and physics, and how to manipulate these to control membrane properties, including membrane asymmetry, binding and fusion at the membrane, or vesicle compartmentalization. The parallel progress in these two areas is now starting to converge, in a manner that synthetic systems that were developed following nature's inspiration are now valuable tools to increase our understanding of natural systems. Therefore, it is not surprising that more and more often researchers are reporting new sophisticated synthetic vesicular systems that can deliver an impact in biological systems, through the controlled (co)administration of drugs, or functioning as synthetic organelles to repair damaged cellular function. This feedback between the fields of natural and synthetic vesicles will open up the path to finally develop applications that were not possible otherwise and eventually replicate and harness the sophisticated functioning of natural cells.

Acknowledgements

The authors acknowledge financial support of this work by the EPSRC (EP/K027263/1) and ERC (Consolidator Grant 614787). FFT acknowledges John Evans and the University of Birmingham for the financial support of the Birmingham Fellowship programme through the John Evans Fellowship.

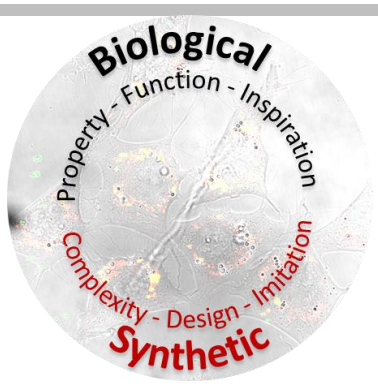
Keywords: biological vesicles • synthetic vesicles • compartmentalization • synthetic cells • polymersomes

10.1002/anie.201607825

WILEY-VCH

REVIEW

While we further our understanding of the biological properties of vesicles and their physiological functions, increasingly elegantly artificial vesicles are being reported for a wide range of technological applications and basic studies. Herein, we bring together both the biological and synthetic state-of-the-art on vesicles and place their biological features in the context of recent synthetic developments, providing a unique view of these complex and rapidly developing fields.



Francisco Fernandez-Trillo, Liam M Grover,* Alex Stephenson-Brown, Paul Harrison, Paula M Mendes,**

*[**Page No. – Page No.***

Vesicles and their multiple facets: underpinning biological and synthetic progress

[1] B. De Kruijff, P. Baken, *Biochim. Biophys. Acta* **1978**, *507*, 38-47.
[2] P. Wolf, *Br. J. Haematol.* **1967**, *13*, 269-288.
[3] H. C. Anderson, *J. Cell. Biol.* **1969**, *41*, 59-72.
[4] R. D. Kornberg, McConnel.Hm, *Biochemistry* **1971**, *10*, 1111-&.
[5] H. T. Cheng, E. London, *Biophys. J.* **2011**, *100*, 2671-2678.
[6] Q. Lin, E. London, *PLoS One* **2014**, *9*.
[7] S.-H. Kim, H. C. Shum, J.-W. Kim, J.-C. Cho, D. A. Weitz, *J. Am. Chem. Soc.* **2011**, *133*, 15165-15171.
[8] S. Pautot, B. J. Frisken, D. A. Weitz, *Proc. Natl. Acad. Sci. U. S. A.* **2003**, *100*, 10718-10721.
[9] R. A. Dragovic, C. Gardiner, A. S. Brooks, D. S. Tannetta, D. J. P. Ferguson, P. Hole, B. Carr, C. W. G. Redman, A. L. Harris, P. J. Dobson, P. Harrison, I. L. Sargent, *Nanomedicine* **2011**, *7*, 780-788.
[10] H. C. Shum, Y.-J. Zhao, S.-H. Kim, D. A. Weitz, *Angew. Chem., Int. Ed.* **2011**, *50*, 1648-1651.
[11] S. Matosevic, B. M. Paegel, *Nat. Chem.* **2013**, *5*, 958-963.
[12] P. D. Robbins, A. E. Morelli, *Nat. Rev. Immunol.* **2014**, *14*, 195-208.
[13] C. D'Souza-Schorey, J. W. Clancy, *Genes Dev.* **2012**, *26*, 1287-1299.
[14] M. J. Hope, T. E. Redelmeier, K. F. Wong, W. Rodrigueza, P. R. Cullis, *Biochemistry* **1989**, *28*, 4181-4187.
[15] M. G. Low, D. B. Zilversmit, *Biochim. Biophys. Acta* **1980**, *596*, 223-234.
[16] D. B. Zilversmit, *J. Lipid Res.* **1984**, *25*, 1563-1569.
[17] K. Sabin, N. Kikyo, *Transl. Res.* **2014**, *163*, 286-295.
[18] A. Czogalla, M. Grzybek, W. Jones, U. Coskun, *Biochim. Biophys. Acta* **2014**, *1841*, 1049-1059.
[19] Y. Nozaki, C. Tanford, *Proc. Natl. Acad. Sci. U. S. A.* **1981**, *78*, 4324-4328.
[20] G. Stengel, R. Zahn, F. Höök, *J. Am. Chem. Soc.* **2007**, *129*, 9584-9585.
[21] A. Musyanovych, K. Landfester, *Macromol. Biosci.* **2014**, *14*, 458-477.
[22] K. Riehemann, S. W. Schneider, T. A. Luger, B. Godin, M. Ferrari, H. Fuchs, *Angew. Chem.-Int. Edit.* **2009**, *48*, 872-897.
[23] X. Guo, F. C. Szoka, *Accounts Chem. Res.* **2003**, *36*, 335-341.
[24] F. Caschera, V. Noireaux, *Curr. Opin. Chem. Biol.* **2014**, *22*, 85-91.
[25] M. M. Hanczyc, J. W. Szostak, *Curr. Opin. Chem. Biol.* **2004**, *8*, 660-664.
[26] P. Walde, *Bioessays* **2010**, *32*, 296-303.
[27] J. P. Luzio, P. R. Pryor, N. A. Bright, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 622-632.
[28] S. Jahne, S. O. Rizzoli, M. S. Helm, *Exp. Cell Res.* **2015**, *335*, 172-179.
[29] F. R. Maxfield, *Cold Spring Harbor Perspect. Biol.* **2014**, *6*, 15.
[30] E. Chargaff, R. West, *J. Biol. Chem.* **1946**, *166*, 189-197.
[31] H. C. Anderson, *J. Cell Biol.* **1969**, *41*, 59-&.
[32] J. Cai, Y. Han, H. M. Ren, C. Y. Chen, D. F. He, L. Zhou, G. M. Eisner, L. D. Asico, P. A. Jose, C. Y. Zeng, *J. Mol. Cell Biol.* **2013**, *5*, 227-238.
[33] K. Ridder, A. Sevko, J. Heide, M. Dams, A.-K. Rupp, J. Macas, J. Starmann, M. Tjwa, K. H. Plate, H. Sueltmann, P. Altevogt, V. Umansky, S. Momma, *Oncoimmunology* **2015**, *4*.
[34] K. Valencia, D. Luis-Ravelo, N. Bovy, I. Anton, S. Martinez-Canarias, C. Zandueta, C. Ormazabal, I. Struman, S. Tabruyn, V. Rebmann, J. De Las Rivas, E. Guruceaga, E. Bandres, F. Lecanda, *Mol. Oncol.* **2014**, *8*, 689-703.
[35] S. B. Walters, J. Kieckbusch, G. Nagalingam, A. Swain, S. L. Latham, G. E. R. Grau, W. J. Britton, V. Combes, B. M. Saunders, *J. Immunol.* **2013**, *190*, 669-677.
[36] S. Sadallah, C. Eken, J. A. Schifferli, *Clin. Exp. Immunol.* **2011**, *163*, 26-32.
[37] J. Park, P. L. Knezevich, W. Wung, S. N. O'Hanlon, A. Goyal, K. L. Benedetti, B. J. Barsi-Rhyne, M. Raman, N. Mock, M. Bremer, M. K. VanHoven, *Neural Dev.* **2011**, *6*.
[38] B. D. Boyan, Z. Schwartz, L. D. Swain, *Bone and Miner.* **1992**, *17*, 263-268.
[39] R. C. Lai, R. W. Y. Yeo, K. H. Tan, S. K. Lim, *Biotechnol. Adv.* **2013**, *31*, 543-551.
[40] R. C. Lai, T. S. Chen, S. K. Lim, *Regen. Med.* **2011**, *6*, 481-492.
[41] T. Lener, M. Gimona, L. Aigner, V. Boerger, E. Buzas, G. Camussi, N. Chaput, D. Chatterjee, F. A. Court, H. A. del Portillo, L. O'Driscoll, S. Fais, J. M. Falcon-Perez, U. Felderhoff-Mueser, L. Fraile, Y. S. Gho, A. Goergens, R. C. Gupta, A. Hendrix, D. M. Hermann, A. F. Hill, F. Hochberg, P. A. Horn, D. de Kleijn, L. Kordelas, B. W. Kramer, E.-M. Kraemer-Albers, S. Laner-Plamberger, S. Laitinen, T. Leonardi, M. J. Lorenowicz, S. K. Lim, J. Lotvall, C. A. Maguire, A. Marcilla, I. Nazarenko, T. Ochiya, T. Patel, S. Pedersen, G. Pocsfalvi, S. Pluchino, P. Quesenberry, I. G. Reischl, F. J. Rivera, R. Sanzenbacher, K. Schallmoser, I. Slaper-Cortenbach, D. Strunk, T. Tonn, P. Vader, B. W. M. van Balkom, M. Wauben, S. El Andaloussi, C. Thery, E. Rohde, B. Giebel, *J. Extracell. Vesicles* **2015**, *4*.
[42] E. van der Pol, A. N. Boing, P. Harrison, A. Sturk, R. Nieuwland, *Pharmacol. Rev.* **2012**, *64*, 676-705.
[43] *Extracellular Vesicles in Health and Disease*, Pan-Stanford Publishing, **2014**.
[44] E. van der Pol, F. A. W. Coumans, A. E. Grootemaat, C. Gardiner, I. L. Sargent, P. Harrison, A. Sturk, T. G. van Leeuwen, R. Nieuwland, *J. Thromb. Haemost.* **2014**, *12*, 1182-1192.

[45] E. Cocucci, J. Meldolesi, *Trends Cell Biol.* **2015**, *25*, 364-372.
[46] E. Cocucci, G. Racchetti, J. Meldolesi, *Trends Cell Biol.* **2009**, *19*, 43-51.
[47] K. W. Witwer, E. I. Buzás, L. T. Bemis, A. Bora, C. Lässer, J. Lötvall, E. N. Nolte-'t Hoen, M. G. Piper, S. Sivaraman, J. Skog, C. Théry, M. H. Wauben, F. Hochberg, *J. Extracell. Vesicles* **2013**.
[48] N. Arraud, R. Linares, S. Tan, C. Gounou, J. M. Pasquet, S. Mornet, A. R. Brisson, *J. Thromb. Haemost.* **2014**, *12*, 614-627.
[49] M. Colombo, C. Moita, G. van Niel, J. Kowal, J. Vigneron, P. Benaroch, N. Manel, L. F. Moita, C. Thery, G. Raposo, *J. Cell Sci.* **2013**, *126*, 5553-5565.
[50] J. H. Hurley, G. Odorizzi, *Nat. Cell Biol.* **2012**, *14*, 654-655.
[51] E. J. Chenette, *Nat. Cell Biol.* **2014**, *16*, 400-400.
[52] G. Raposo, M. N. Cordonnier, D. Tenza, B. Menichi, A. Durrbach, D. Louvard, E. Coudrier, *Mol. Biol. Cell* **1999**, *10*, 1477-1494.
[53] T. Kobayashi, F. Gu, J. Gruenberg, *Semin. Cell Dev. Biol.* **1998**, *9*, 517-526.
[54] M. Ostrowski, N. B. Carmo, S. Krumeich, I. Fanget, G. Raposo, A. Savina, C. F. Moita, K. Schauer, A. N. Hume, R. P. Freitas, B. Goud, P. Benaroch, N. Hacohen, M. Fukuda, C. Desnos, M. C. Seabra, F. Darchen, S. Amigorena, L. F. Moita, C. Thery, *Nat. Cell Biol.* **2010**, *12*, 19-U61.
[55] C. Hsu, Y. Morohashi, S.-i. Yoshimura, N. Manrique-Hoyos, S. Jung, M. A. Lauterbach, M. Bakhti, M. Gronborg, W. Moebius, J. Rhee, F. A. Barr, M. Simons, *J. Cell Biol.* **2010**, *189*, 223-232.
[56] Y. A. Chen, R. H. Scheller, *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 98-106.
[57] G. Raposo, W. Stoorvogel, *J. Cell Biol.* **2013**, *200*, 373-383.
[58] A. Piccin, W. G. Murphy, O. P. Smith, *Blood Rev.* **2007**, *21*, 157-171.
[59] J. Rak, *Semin. Thromb. Hemost.* **2010**, *36*, 888-906.
[60] G. T. Szabo, B. Tarr, K. Paloczi, K. Eder, E. Lajko, A. Kittel, S. Toth, B. Gyorgy, M. Pasztoi, A. Nemeth, X. Osteikoetxea, E. Pallinger, A. Falus, K. Szabo-Taylor, E. I. Buzas, *Cell. Mol. Life Sci.* **2014**, *71*, 4055-4067.
[61] H. Kalra, G. P. C. Drummen, S. Mathivanan, *Int. J. Mol. Sci.* **2016**, *17*.
[62] L. Holmgren, A. Szeles, E. Rajnavolgyi, J. Folkman, G. Klein, I. Ernberg, K. I. Falk, *Blood* **1999**, *93*, 3956-3963.
[63] M. Valapala, J. K. Vishwanatha, *J. Biol. Chem.* **2011**, *286*, 30911-30925.
[64] J. H. Hurley, E. Boura, L. A. Carlson, B. Rozycki, *Cell* **2010**, *143*, 875-887.
[65] B. Fadeel, D. Xue, **2009**, *44*, 264-277.
[66] M. Bogdanov, W. Dowhan, H. Vitrac, *Biochim. Biophys. Acta-Mol. Cell Res.* **2014**, *1843*, 1475-1488.
[67] G. Pocsfalvi, C. Stanly, A. Vilasi, I. Fiume, G. Capasso, L. Turiak, E. I. Buzas, K. Vekey, *Mass Spectrom. Rev.* **2016**, *35*, 3-21.
[68] S. Kreimer, A. M. Belov, I. Ghiran, S. K. Murthy, D. A. Frank, A. R. Ivanov, *J. Proteome Res.* **2015**, *14*, 2367-2384.
[69] A. de Gassart, C. Geminard, B. Fevrier, G. Raposo, M. Vidal, *Blood* **2003**, *102*, 4336-4344.
[70] K. Laulagnier, C. Motta, S. Hamdi, S. Roy, F. Fauvelle, J. F. Pageaux, T. Kobayashi, J. P. Salles, B. Perret, C. Bonnerot, M. Record, *Biochem. J.* **2004**, *380*, 161-171.
[71] H. Kalra, C. G. Adda, M. Liem, C. S. Ang, A. Mechler, R. J. Simpson, M. D. Hulett, S. Mathivanan, *Proteomics* **2013**, *13*, 3354-3364.
[72] C. Subra, K. Laulagnier, B. Perret, M. Record, *Biochimie* **2007**, *89*, 205-212.
[73] C. Thery, M. Boussac, P. Veron, P. Ricciardi-Castagnoli, G. Raposo, J. Garin, S. Amigorena, *J. Immunol.* **2001**, *166*, 7309-7318.
[74] M. Vidal, J. Saintemarie, J. R. Philippot, A. Bienvenue, *J. Cell. Physiol.* **1989**, *140*, 455-462.
[75] Y. Yu, J. A. Vroman, S. C. Bae, S. Granick, *J. Am. Chem. Soc.* **2010**, *132*, 195-201.
[76] L. Li, J. X. Cheng, *Biochemistry* **2006**, *45*, 11819-11826.
[77] M. Yanagisawa, M. Imai, T. Taniguchi, *Phys. Rev. Lett.* **2008**, *100*, 4.
[78] P. A. Beales, T. K. Vanderlick, *J. Phys. Chem. B* **2009**, *113*, 13678-13686.
[79] M. Loew, R. Springer, S. Scolari, F. Altenbrunn, O. Seitz, J. Liebscher, D. Huster, A. Herrmann, A. Arbuzova, *J. Am. Chem. Soc.* **2010**, *132*, 16066-16072.
[80] J. H. Fuhrhop, D. Fritsch, *Accounts Chem. Res.* **1986**, *19*, 130-137.
[81] X. M. Lian, D. X. Wu, X. H. Song, H. Y. Zhao, *Macromolecules* **2010**, *43*, 7434-7445.
[82] A. Blanazs, M. Massignani, G. Battaglia, S. P. Armes, A. J. Ryan, *Adv. Funct. Mater.* **2009**, *19*, 2906-2914.
[83] Y. Dong, Y. Sun, L. Wang, D. Wang, T. Zhou, Z. Yang, Z. Chen, Q. Wang, Q. Fan, D. Liu, *Angew. Chem., Int. Ed.* **2014**, n/a-n/a.
[84] Q. M. Liu, J. Chen, J. Z. Du, *Biomacromolecules* **2014**, *15*, 3072-3082.
[85] A. Uesaka, M. Ueda, T. Imai, J. Sugiyama, S. Kimura, *Langmuir* **2014**, *30*, 4273-4279.
[86] S. Pautot, B. J. Frisken, D. A. Weitz, *Langmuir* **2003**, *19*, 2870-2879.
[87] E. Greco, G. Quintiliani, M. B. Santucci, A. Serafino, A. R. Ciccaglione, C. Marcantonio, M. Papi, G. Maulucci, G. Delogu, A. Martino, D. Goletti, L. Sarmati, M. Andreoni, A. Altieri, M. Alma, N. Caccamo, D. Di Liberto, M. De Spirito, N. D. Savage, R. Nisini, F. Dieli, T. H. Ottenhoff, M. Fraziano, *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, E1360-E1368.
[88] H. Vitrac, M. Bogdanov, W. Dowhan, *Proc. Natl. Acad. Sci. U. S. A.* **2013**, *110*, 9338-9343.
[89] R. Tunuguntla, M. Bangar, K. Kim, P. Stroeve, C. M. Ajo-Franklin, A. Noy, *Biophys. J.* **2013**, *105*, 1388-1396.
[90] M. Yanagisawa, M. Iwamoto, A. Kato, K. Yoshikawa, S. Oiki, *J. Am. Chem. Soc.* **2011**, *133*, 11774-11779.
[91] M. Son, E. London, *J. Lipid Res.* **2013**, *54*, 3385-3393.
[92] C. Corrado, S. Raimondo, A. Chiesi, F. Ciccia, G. De Leo, R. Alessandro, *Int. J. Mol. Sci.* **2013**, *14*, 5338-5366.
[93] A. Clayton, A. Turkes, S. Dewitt, R. Steadman, M. D. Mason, M. B. Hallett, *Faseb J.* **2004**, *18*, 977-+.
[94] A. Calzolari, C. Raggi, S. Deaglio, N. M. Sposi, M. Stafsnes, K. Fecchi, I. Parolini, F. Malavasi, C. Peschle, M. Sargiacomo, U. Testa, *J. Cell Sci.* **2006**, *119*, 4486-4498.

[95] A. Clayton, J. P. Mitchell, J. Court, S. Linnane, M. D. Mason, Z. Tabi, *J. Immunol.* **2008**, *180*, 7249-7258.
[96] S. Rana, M. Zoller, *Biochem. Soc. Trans.* **2011**, *39*, 559-562.
[97] S. Levy, T. Shoham, *Physiology* **2005**, *20*, 218-224.
[98] T. Tian, Y. L. Zhu, Y. Y. Zhou, G. F. Liang, Y. Y. Wang, F. H. Hu, Z. D. Xiao, *J. Biol. Chem.* **2014**, *289*, 22258-22267.
[99] K. J. Svensson, H. C. Christianson, A. Wittrup, E. Bourseau-Guilmain, E. Lindqvist, L. M. Svensson, M. Morgelin, M. Belting, *J. Biol. Chem.* **2013**, *288*, 17713-17724.
[100] M. Miyanishi, K. Tada, M. Koike, Y. Uchiyama, T. Kitamura, S. Nagata, *Nature* **2007**, *450*, 435-439.
[101] J. Voskuhl, B. J. Ravoo, *Chem. Soc. Rev.* **2009**, *38*, 495-505.
[102] C. W. Lim, B. J. Ravoo, D. N. Reinhoudt, *Chem. Commun.* **2005**, 5627-5629.
[103] H. K. Lee, K. M. Park, Y. J. Jeon, D. Kim, D. H. Oh, H. S. Kim, C. K. Park, K. Kim, *J. Am. Chem. Soc.* **2005**, *127*, 5006-5007.
[104] B. Gruber, S. Balk, S. Stadlbauer, B. Konig, *Angew. Chem.-Int. Edit.* **2012**, *51*, 10060-10063.
[105] S. Banerjee, M. Bhuyan, B. Konig, *Chem. Commun.* **2013**, *49*, 5681-5683.
[106] A. Muller, B. Konig, *Chem. Commun.* **2014**, *50*, 12665-12668.
[107] A. Hennig, G. Ghale, W. M. Nau, *Chem. Commun.* **2007**, 1614-1616.
[108] J. Kikuchi, K. Ariga, K. Ikeda, *Chem. Commun.* **1999**, 547-548.
[109] R. J. R. W. Peters, M. Nijemeisland, J. C. M. van Hest, *Angew. Chem. Int. Ed.* **2015**, *54*, 9614-9617.
[110] K. Bernitzki, T. Schrader, *Angew. Chem. Int. Ed.* **2009**, *48*, 8001-8005.
[111] H. P. Dijkstra, J. J. Hutchinson, C. A. Hunter, H. Qin, S. Tomas, S. J. Webb, N. H. Williams, *Chem. Eur. J.* **2007**, *13*, 7215-7222.
[112] P. Barton, C. A. Hunter, T. J. Potter, S. J. Webb, N. H. Williams, *Angew. Chem.-Int. Edit.* **2002**, *41*, 3878-3881.
[113] K. Bernitzki, M. Maue, T. Schrader, *Chem. Eur. J.* **2012**, *18*, 13412-13417.
[114] H. R. Marsden, I. Tomatsu, A. Kros, *Chem. Soc. Rev.* **2011**, *40*, 1572-1585.
[115] C. M. Paleos, D. Tsiourvas, Z. Sideratou, *ChemBioChem* **2011**, *12*, 510-521.
[116] G. Stengel, R. Zahn, F. Hook, *J. Am. Chem. Soc.* **2007**, *129*, 9584–9585.
[117] D. Papahadjopoulos, S. Nir, N. Duzgunes, *J. Bioenerg. Biomembr.* **1990**, *22*, 157-179.
[118] A. Richard, V. Marchi-Artzner, M. N. Lalloz, M. J. Brienne, F. Artzner, T. Gulik-Krzywicki, M. A. Guedeau-Boudeville, J. M. Lehn, *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 15279-15284.
[119] H. B. Jin, Y. Liu, Y. L. Zheng, W. Huang, Y. F. Zhou, D. Y. Yan, *Langmuir* **2012**, *28*, 2066-2072.
[120] F. Versluis, J. Voskuhl, J. Vos, H. Friedrich, B. J. Ravoo, P. H. H. Bomans, M. C. A. Stuart, N. A. J. M. Sommerdijk, A. Kros, *Soft Matter* **2014**, *10*, 9746-9751.
[121] A. Kashiwada, M. Tsuboi, K. Matsuda, *Chem. Commun.* **2009**, 695-697.
[122] H.-P. M. de Hoog, M. Nallani, N. Tomczak, *Soft Matter* **2012**, *8*, 4552-4561.
[123] C. M. Paleos, D. Tsiourvas, Z. Sideratou, *Langmuir* **2012**, *28*, 2337-2346.
[124] M. Marguet, C. Bonduelle, S. Lecommandoux, *Chem. Soc. Rev.* **2013**, *42*, 512-529.
[125] L. M. Traub, *Biochim. Biophys. Acta* **2005**, *1744*, 415-437.
[126] S. Mayor, R. E. Pagano, *Nat. Rev. Mol. Cell Biol.* **2007**, *8*, 603-612.
[127] T. Kirchhausen, *Nat. Rev. Mol. Cell Biol.* **2000**, *1*, 187-198.
[128] J. S. Bonifacino, B. S. Glick, *Cell* **2004**, *116*, 153-166.
[129] H. Hotani, *J. Mol. Biol.* **1984**, *178*, 113-120.
[130] T. Hamada, Y. Miura, K.-i. Ishii, S. Araki, K. Yoshikawa, M. d. Vestergaard, M. Takagi, *J. Phys. Chem. B* **2007**, *111*, 10853-10857.
[131] Y. Okumura, T. Nakaya, H. Namai, K. Urita, *Langmuir* **2011**, *27*, 3279-3282.
[132] P.-Y. Bolinger, D. Stamou, H. Vogel, *Angew. Chem., Int. Ed.* **2008**, *47*, 5544-5549.
[133] P.-Y. Bolinger, D. Stamou, H. Vogel, *J. Am. Chem. Soc.* **2004**, *126*, 8594-8595.
[134] S. A. Walker, M. T. Kennedy, J. A. Zasadzinski, *Nature* **1997**, *387*, 61-64.
[135] E. T. Kisak, B. Coldren, J. A. Zasadzinski, *Langmuir* **2002**, *18*, 284-288.
[136] C. Boyer, J. A. Zasadzinski, *ACS Nano* **2007**, *1*, 176-182.
[137] C. M. Paleos, A. Pantos, *Acc. Chem. Res.* **2014**, *47*, 1475-1482, and references therein.
[138] W. J. Duncanson, T. Lin, A. R. Abate, S. Seiffert, R. K. Shah, D. A. Weitz, *Lab Chip* **2012**, *12*, 2135-2145.
[139] W. T. Al-Jamal, K. Kostarelos, *Int. J. Pharm.* **2007**, *331*, 182-185.
[140] E. Lorenceau, A. S. Utada, D. R. Link, G. Cristobal, M. Joanicot, D. A. Weitz, *Langmuir* **2005**, *21*, 9183-9186.
[141] M. Marguet, L. Edembe, S. Lecommandoux, *Angew. Chem., Int. Ed.* **2012**, *51*, 1173-1176.
[142] D. M. Vriezema, M. Comellas Aragonès, J. A. A. W. Elemans, J. J. L. M. Cornelissen, A. E. Rowan, R. J. M. Nolte, *Chem. Rev.* **2005**, *105*, 1445-1489.
[143] H. Chaimovich, I. M. Cuccovia, in *Amphiphiles at Interfaces, Vol. 103*, Steinkopff, Darmstadt, **1997**, pp. 67-77.
[144] R. J. Ellis, *Trends Biochem. Sci.* **2001**, *26*, 597-604.
[145] M. Yoshimoto, *Methods Mol. Biol.* **2011**, *679*, 9-18.
[146] J. Van Esch, M. F. M. Roks, R. J. M. Nolte, *J. Am. Chem. Soc.* **1986**, *108*, 6093-6094.
[147] G. M. Cooper, R. E. Hausman, in *The cell : a molecular approach*, ASM Press ; Sinauer Associates, Washington, D.C.; Sunderland, Mass., **2009**.
[148] M. Bloom, E. Evans, O. G. Mouritsen, *Q. Rev. Biophys.* **1991**, *24*, 293-397.
[149] E. A. Disalvo, S. A. Simon, *Permeability and stability of lipid bilayers*, CRC Press, Boca Raton, **1995**.

[150] T. J. McIntosh, in *Current Topics in Membranes, Vol. Volume 48* (Eds.: A. K. Davld W. Deamer, M. F. Douglas), Academic Press, **1999**, pp. 23-47.
[151] D. Marsh, *Handbook of lipid bilayers*, Boca Raton, FL, CRC Press, **2013**.
[152] D. Needham, R. S. Nunn, *Biophys. J.* **1990**, *58*, 997-1009.
[153] I. van Uitert, S. Le Gac, A. van den Berg, *Biochim. Biophys. Acta* **2010**, *1798*, 21-31.
[154] M. C. Woodle, D. D. Lasic, *Biochim. Biophys. Acta* **1992**, *1113*, 171-199.
[155] D. Marsh, R. Bartucci, L. Sportelli, *Biochim. Biophys. Acta* **2003**, *1615*, 33-59.
[156] P. Aleksandr, K. Alexander, D. R. Robert, P. T. Vladimir, in *Liposomes, Lipid Bilayers and Model Membranes*, CRC Press, **2014**, pp. 317-340.
[157] K. T. Kim, S. A. Meeuwissen, R. J. M. Nolte, J. C. M. van Hest, *Nanoscale* **2010**, *2*, 844-858.
[158] K. Renggli, P. Baumann, K. Langowska, O. Onaca, N. Bruns, W. Meier, *Adv. Funct. Mater.* **2011**, *21*, 1241-1259.
[159] P. Tanner, S. Egli, V. Balasubramanian, O. Onaca, C. G. Palivan, W. Meier, *FEBS Lett.* **2011**, *585*, 1699-1706.
[160] D. E. Discher, F. Ahmed, *Annu. Rev. Biomed. Eng.* **2006**, *8*, 323-341.
[161] C. Lopresti, H. Lomas, M. Massignani, T. Smart, G. Battaglia, *J. Mater. Chem.* **2009**, *19*, 3576.
[162] A. Koide, A. Kishimura, K. Osada, W.-D. Jang, Y. Yamasaki, K. Kataoka, *J. Am. Chem. Soc.* **2006**, *128*, 5988-5989.
[163] B. Le Droumaguet, K. Velonia, *Angew. Chem., Int. Ed.* **2008**, *47*, 6263-6266.
[164] P. Horcajada, T. Chalati, C. Serre, B. Gillet, C. Sebrie, T. Baati, J. F. Eubank, D. Heurtaux, P. Clayette, C. Kreuz, J.-S. Chang, Y. K. Hwang, V. Marsaud, P.-N. Bories, L. Cynober, S. Gil, G. Ferey, P. Couvreur, R. Gref, *Nat. Mater.* **2010**, *9*, 172-178.
[165] J. F. Le Meins, O. Sandre, S. Lecommandoux, *Eur. Phys. J. E* **2011**, *34*, 1-17.
[166] C. Nardin, S. Thoeni, J. Widmer, M. Winterhalter, W. Meier, *Chem. Commun.* **2000**, 1433-1434.
[167] A. Ranquin, W. Versées, W. Meier, J. Steyaert, P. van Gelder, *Nano Lett.* **2005**, *5*, 2220-2224.
[168] P. Tanner, O. Onaca, V. Balasubramanian, W. Meier, C. G. Palivan, *Chem.-Eur. J.* **2011**, *17*, 4552-4560.
[169] C. De Vocht, A. Ranquin, R. Willaert, J. A. Van Ginderachter, T. Vanhaecke, V. Rogiers, W. Versées, P. van Gelder, J. Steyaert, *J. Controlled Release* **2009**, *137*, 246-254.
[170] V. Noireaux, A. Libchaber, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 17669-17674.
[171] K. T. Kim, J. J. L. M. Cornelissen, R. J. M. Nolte, J. C. M. van Hest, *Adv. Mater.* **2009**, *21*, 2787-2791.
[172] J. Gaitzsch, D. Appelhans, L. Wang, G. Battaglia, B. Voit, *Angew. Chem., Int. Ed.* **2012**, *51*, 4448-4451.
[173] D. Gräfe, J. Gaitzsch, D. Appelhans, B. Voit, *Nanoscale* **2014**, *6*, 10752-10761.
[174] Q. Yan, J. Wang, Y. Yin, J. Yuan, *Angew. Chem., Int. Ed.* **2013**, *52*, 5070-5073.
[175] M. V. Dinu, M. Spulber, K. Renggli, D. Wu, C. A. Monnier, A. Petri-Fink, N. Bruns, *Macromol. Rapid Comm.* **2015**.
[176] S. Stolpe, T. Friedrich, *J. Biol. Chem.* **2004**, *279*, 18377-18383.
[177] A. Graff, C. Fraysse-Ailhas, C. G. Palivan, M. Grzelakowski, T. Friedrich, C. Vebert, G. Gescheidt, W. Meier, *Macromol. Chem. Phys.* **2010**, *211*, 229-238.
[178] D. M. Vriezema, P. M. L. Garcia, N. Sancho Oltra, N. S. Hatzakis, S. M. Kuiper, R. J. M. Nolte, A. E. Rowan, J. C. M. van Hest, *Angew. Chem., Int. Ed. Engl.* **2007**, *46*, 7378-7382.
[179] S. F. M. van Dongen, M. Nallani, J. L. L. M. Cornelissen, R. J. M. Nolte, J. C. M. van Hest, *Chem.-Eur. J.* **2009**, *15*, 1107-1114.
[180] R. J. R. W. Peters, M. Marguet, S. Marais, M. W. Fraaije, J. C. M. van Hest, S. Lecommandoux, *Angew. Chem., Int. Ed.* **2014**, *53*, 146-150.
[181] S. M. Kuiper, M. Nallani, D. M. Vriezema, J. J. L. M. Cornelissen, J. C. M. van Hest, R. J. M. Nolte, A. E. Rowan, *Org. Biomol. Chem.* **2008**, *6*, 4315-4318.
[182] G. Delaittre, I. C. Reynhout, J. J. L. M. Cornelissen, R. J. M. Nolte, *Chem.-Eur. J.* **2009**, *15*, 12600-12603.
[183] S. A. Meeuwissen, A. Rioz-Martínez, G. de Gonzalo, M. W. Fraaije, V. Gotor, J. C. M. van Hest, *J. Mater. Chem.* **2011**, *21*, 18923.
[184] I. Louzao, J. C. M. van Hest, *Biomacromolecules* **2013**, *14*, 2364-2372.
[185] M. N. Jones, K. J. Hill, M. Kaszuba, J. E. Creeth, *Int. J. Pharm.* **1998**, *162*, 107-117.
[186] R. Roodbeen, J. C. M. van Hest, *Bioessays* **2009**, *31*, 1299-1308.
[187] R. J. R. W. Peters, I. Louzao, J. C. M. van Hest, *Chem Sci* **2012**, *3*, 335-342.
[188] N. Ben-Haim, P. Broz, S. Marsch, W. Meier, P. Hunziker, *Nano Lett.* **2008**, *8*, 1368-1373.
[189] G. Steinberg-Yfrach, J. L. Rigaud, E. N. Durantini, A. L. Moore, D. Gust, T. A. Moore, *Nature* **1998**, *392*, 479-482.
[190] P. Richard, J. L. Rigaud, P. Gräber, *Eur. J. Biochem.* **1990**, *193*, 921-925.
[191] H.-J. Choi, C. D. Montemagno, *Nano Lett.* **2005**, *5*, 2538-2542.
[192] P. Stano, P. L. Luisi, *Chem. Commun.* **2010**, *46*, 3639.
[193] M. Forlin, R. Lentini, S. S. Mansy, *Curr. Opin. Chem. Biol.* **2012**, *16*, 586-592.
[194] J. C. Blain, J. W. Szostak, *Annu. Rev. Biochem.* **2014**, *83*, 615-640.
[195] L. Cronin, N. Krasnogor, B. G. Davis, C. Alexander, N. Robertson, J. H. G. Steinke, S. L. M. Schroeder, A. N. Khlobystov, G. Cooper, P. M. Gardner, P. Siepmann, B. J. Whitaker, D. Marsh, *Nat. Biotechnol.* **2006**, *24*, 1203-1206.
[196] P. K. Schmidli, P. Schurtenberger, P. L. Luisi, *J. Am. Chem. Soc.* **1991**, *113*, 8127-8130.
[197] P. Walde, A. Goto, P.-A. Monnard, M. Wessicken, P. L. Luisi, *J. Am. Chem. Soc.* **1994**, *116*, 7541-7547.
[198] P. Walde, R. Wick, M. Fresta, A. Mangone, P. L. Luisi, *J. Am. Chem. Soc.* **1994**, *116*, 11649-11654.
[199] S. S. Mansy, J. P. Schrum, M. Krishnamurthy, S. Tobé, D. A. Treco, J. W. Szostak, *Nature* **2008**, *454*, 122-125.
[200] T. F. Zhu, J. W. Szostak, *J. Am. Chem. Soc.* **2009**, *131*, 5705-5713.
[201] K. Kurihara, M. Tamura, K.-i. Shohda, T. Toyota, K. Suzuki, T. Sugawara, *Nat. Chem.* **2011**, *3*, 775-781.
[202] G. B. Ogden, M. J. Pratt, M. Schaechter, *Cell* **1988**, *54*, 127-135.

10.1002/anie.201607825